

## ENZYMES HAVING ALPHA AMYLASE ACTIVITY AND METHODS OF USE THEREOF

### RELATED APPLICATION DATA

[0001] This application claims priority of U.S. Provisional Application No. 60/270,495, filed February 21, 2001, now pending; U.S. Provisional Application No. 60/270,496, filed February 21, 2001, now pending; and U.S. Provisional Application No. 60/291,122, filed May 14, 2001, now pending, all of which are herein incorporated by reference in their entirety.

### FIELD OF THE INVENTION

[0002] This invention relates generally to enzymes, polynucleotides encoding the enzymes, the use of such polynucleotides and polypeptides, and more specifically to enzymes having alpha amylase activity.

### BACKGROUND

[0003] Starch is a complex carbohydrate often found in the human diet. The structure of starch is glucose polymers linked by  $\alpha$ -1,4 and  $\alpha$ -1,6 glucosidic bonds. Amylase is an enzyme that catalyzes the hydrolysis of starches into sugars. Amylases hydrolyze internal  $\alpha$ -1,4-glucosidic linkages in starch, largely at random, to produce smaller molecular weight malto-dextrins. The breakdown of starch is important in the digestive system and commercially. Amylases are of considerable commercial value, being used in the initial stages (liquefaction) of starch processing; in wet corn milling; in alcohol production; as cleaning agents in detergent matrices; in the textile industry for starch desizing; in baking applications; in the beverage industry; in oilfields in drilling processes; in inking of recycled paper; and in animal feed.

[0004] Amylases are produced by a wide variety of microorganisms including *Bacillus* and *Aspergillus*, with most commercial amylases being produced from bacterial sources such as *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus subtilis*, or

*Bacillus stearothermophilus*. In recent years, the enzymes in commercial use have been those from *Bacillus licheniformis* because of their heat stability and performance, at least at neutral and mildly alkaline pHs.

[0005] In general, starch to fructose processing consists of four steps: liquefaction of granular starch, saccharification of the liquefied starch into dextrose, purification, and isomerization to fructose. The object of a starch liquefaction process is to convert a concentrated suspension of starch polymer granules into a solution of soluble shorter chain length dextrans of low viscosity. This step is essential for convenient handling with standard equipment and for efficient conversion to glucose or 10<sup>3</sup> other sugars. To liquefy granular starch, it is necessary to gelatinize the granules by raising the temperature of the granular starch to over about 72°C. The heating process instantaneously disrupts the insoluble starch granules to produce a water soluble starch solution. The solubilized starch solution is then liquefied by amylase. A starch granule is composed of: 69-74% amylopectin, 26-31% amylose, 11-14% water, 0.2-0.4% protein, 0.5-0.9% lipid, 0.05-0.1% ash, 0.02-0.03% phosphorus, 0.1% pentosan. Approximately 70% of a granule is amorphous and 30% is crystalline.

[0006] A common enzymatic liquefaction process involves adjusting the pH of a granular starch slurry to between 6.0 and 6.5, the pH optimum of alpha-amylase derived from *Bacillus licheniformis*, with the addition of calcium hydroxide, sodium hydroxide or sodium carbonate. The addition of calcium hydroxide has the advantage of also providing calcium ions which are known to stabilize the alpha-amylase against inactivation. Upon addition of alpha-amylase, the suspension is pumped through a steam jet to instantaneously raise the temperature to between 80 degree-115 degrees C. The starch is immediately gelatinized and, due to the presence of alpha-amylase, depolymerized through random hydrolysis of a (1-4) glycosidic bonds by alpha-amylase to a fluid mass which is easily pumped.

[0007] In a second variation to the liquefaction process, alpha-amylase is added to the starch suspension, the suspension is held at a temperature of 80-100 degrees C to partially hydrolyze the starch granules, and the partially hydrolyzed starch suspension is pumped through a jet at temperatures in excess of about 105 degrees C to thoroughly gelatinize any

remaining granular structure. After cooling the gelatinized starch, a second addition of alpha-amylase can be made to further hydrolyze the starch.

**[0008]** A third variation of this process is called the dry milling process. In dry milling, whole grain is ground and combined with water. The germ is optionally removed by flotation separation or equivalent techniques. The resulting mixture, which contains starch, fiber, protein and other components of the grain, is liquefied using  $\alpha$ -amylase. The general practice in the art is to undertake enzymatic liquefaction at a lower temperature when using the dry milling process. Generally, low temperature liquefaction is believed to be less efficient than high temperature liquefaction in converting starch to soluble dextrins.

**[0009]** Typically, after gelatinization the starch solution is held at an elevated temperature in the presence of alpha-amylase until a DE of 10-20 is achieved, usually a period of 1-3 hours. Dextrose equivalent (DE) is the industry standard for measuring the concentration of total reducing sugars, calculated as D-glucose on a dry weight basis. Unhydrolyzed granular starch has a DE of virtually zero, whereas the DE of D-glucose is defined as 100.

**[0010]** Corn wet milling is a process which produces corn oil, gluten meal, gluten feed and starch. Alkaline-amylase is used in the liquefaction of starch and glucoamylase is used in saccharification, producing glucose. Corn, a kernel of which consists of a outer seed coat (fiber), starch, a combination of starch and glucose and the inner germ, is subjected to a four step process, which results in the production of starch. The corn is steeped, de-germed, de-fibered, and finally the gluten is separated. In the steeping process, the solubles are taken out. The product remaining after removal of the solubles is de-germed, resulting in production of corn oil and production of an oil cake, which is added to the solubles from the steeping step. The remaining product is de-fibered and the fiber solids are added to the oil cake/solubles mixture. This mixture of fiber solids, oil cake and solubles forms a gluten feed. After de-fibered, the remaining product is subjected to gluten separation. This separation results in a gluten meal and starch. The starch is then subjected to liquefaction and saccharification to produce glucose.

**[0011]** Staling of baked products (such as bread) has been recognized as a problem which becomes more serious as more time lies between the moment of preparation of the

bread product and the moment of consumption. The term staling is used to describe changes undesirable to the consumer in the properties of the bread product after leaving the oven, such as an increase of the firmness of the crumb, a decrease of the elasticity of the crumb, and changes in the crust, which becomes tough and leathery. The firmness of the bread crumb increases further during storage up to a level, which is considered as negative. The increase in crumb firmness, which is considered as the most important aspect of staling, is recognized by the consumer a long time before the bread product has otherwise become unsuitable for consumption.

[0012] There is a need in the industry for the identification and optimization of amylases, useful for various uses, including commercial cornstarch liquefaction processes. These second generation acid amylases will offer improved manufacturing and/or performance characteristics over the industry standard enzymes from *Bacillus licheniformis*, for example.

[0013] There is also a need for the identification and optimization of amylases having utility in automatic dish wash (ADW) products and laundry detergent. In ADW products, the amylase will function at pH 10-11 and at 45-60 °C in the presence of calcium chelators and oxidative conditions. For laundry, activity at pH 9-10 and 40 °C in the appropriate detergent matrix will be required. Amylases are also useful in textile desizing, brewing processes, starch modification in the paper and pulp industry and other processes described in the art.

[0014] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

## SUMMARY OF THE INVENTION

[0015] The invention provides an isolated nucleic acid having a sequence as set forth in SEQ ID Nos.: 1, 3, 5, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203,



205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 251, 253, 255, 257, 259, 261, 263, 265, 267, 269, 271, 273, 275, 277, 279, 281, 283, 285, 287, 289, 291, 293, 295, 297, 299 and variants thereof having at least 50% sequence identity to SEQ ID Nos.: 1, 3, 5, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 251, 253, 255, 257, 259, 261, 263, 265, 267, 269, 271, 273, 275, 277, 279, 281, 283, 285, 287, 289, 291, 293, 295, 297, 299 and encoding polypeptides having alpha amylase activity.

**[0016]** One aspect of the invention is an isolated nucleic acid having a sequence as set forth in SEQ ID Nos: 1, 3, 5, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 251, 253, 255, 257, 259, 261, 263, 265, 267, 269, 271, 273, 275, 277, 279, 281, 283, 285, 287, 289, 291, 293, 295, 297, 299 (hereinafter referred to as "Group A nucleic acid sequences"), sequences substantially identical thereto, and sequences complementary thereto.

**[0017]** Another aspect of the invention is an isolated nucleic acid including at least 10 consecutive bases of a sequence as set forth in Group A nucleic acid sequences, sequences substantially identical thereto, and the sequences complementary thereto.

**[0018]** In yet another aspect, the invention provides an isolated nucleic acid encoding a polypeptide having a sequence as set forth in SEQ ID Nos.: 2, 4, 6, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114,

116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 252, 254, 256, 258, 260, 262, 264, 266, 268, 270, 272, 274, 276, 278, 280, 282, 284, 286, 288, 290, 292, 294, 296, 298 and variants thereof encoding a polypeptide having alpha amylase activity and having at least 50% sequence identity to such sequences.

[0019] Another aspect of the invention is an isolated nucleic acid encoding a polypeptide or a functional fragment thereof having a sequence as set forth in SEQ ID No.s: 2, 4, 6, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 252, 254, 256, 258, 260, 262, 264, 266, 268, 270, 272, 274, 276, 278, 280, 282, 284, 286, 288, 290, 292, 294, 296, 298 (hereinafter referred to as "Group B amino acid sequences"), and sequences substantially identical thereto.

[0020] Another aspect of the invention is an isolated nucleic acid encoding a polypeptide having at least 10 consecutive amino acids of a sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto.

[0021] In yet another aspect, the invention provides a purified polypeptide having a sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto.

[0022] Another aspect of the invention is an isolated or purified antibody that specifically binds to a polypeptide having a sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto.

[0023] Another aspect of the invention is an isolated or purified antibody or binding fragment thereof, which specifically binds to a polypeptide having at least 10 consecutive

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amino acids of one of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto.

[0024] Another aspect of the invention is a method of making a polypeptide having a sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto. The method includes introducing a nucleic acid encoding the polypeptide into a host cell, wherein the nucleic acid is operably linked to a promoter, and culturing the host cell under conditions that allow expression of the nucleic acid.

[0025] Another aspect of the invention is a method of making a polypeptide having at least 10 amino acids of a sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto. The method includes introducing a nucleic acid encoding the polypeptide into a host cell, wherein the nucleic acid is operably linked to a promoter, and culturing the host cell under conditions that allow expression of the nucleic acid, thereby producing the polypeptide.

[0026] Another aspect of the invention is a method of generating a variant including obtaining a nucleic acid having a sequence as set forth in Group A nucleic acid sequences, sequences substantially identical thereto, sequences complementary to the sequences of Group A nucleic acid sequences, fragments comprising at least 30 consecutive nucleotides of the foregoing sequences, and changing one or more nucleotides in the sequence to another nucleotide, deleting one or more nucleotides in the sequence, or adding one or more nucleotides to the sequence.

[0027] Another aspect of the invention is a computer readable medium having stored thereon a sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto.

[0028] Another aspect of the invention is a computer system including a processor and a data storage device wherein the data storage device has stored thereon a sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide having a sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto.

[0029] Another aspect of the invention is a method for comparing a first sequence to a reference sequence wherein the first sequence is a nucleic acid having a sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide code of Group B amino acid sequences, and sequences substantially identical thereto. The method includes reading the first sequence and the reference sequence through use of a computer program which compares sequences; and determining differences between the first sequence and the reference sequence with the computer program.

[0030] Another aspect of the invention is a method for identifying a feature in a sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide having a sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto, including reading the sequence through the use of a computer program which identifies features in sequences; and identifying features in the sequence with the computer program.

[0031] Another aspect of the invention is an assay for identifying fragments or variants of Group B amino acid sequences, and sequences substantially identical thereto, which retain the enzymatic function of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto. The assay includes contacting the polypeptide of Group B amino acid sequences, sequences substantially identical thereto, or polypeptide fragment or variant with a substrate molecule under conditions which allow the polypeptide fragment or variant to function, and detecting either a decrease in the level of substrate or an increase in the level of the specific reaction product of the reaction between the polypeptide and substrate thereby identifying a fragment or variant of such sequences.

[0032] The invention also provides a process for preparing a dough or a baked product prepared from the dough which comprises adding an amylase of the invention to the dough in an amount which is effective to retard the staling of the bread. The invention also provides a dough comprising said amylase and a premix comprising flour together with said amylase. Finally, the invention provides an enzymatic baking additive, which contains said amylase.

[0033] The use of the amylase in accordance with the present invention provides an improved anti-staling effect as measured by, e.g. less crumb firming, retained crumb

elasticity, improved slice-ability (e.g. fewer crumbs, non-gummy crumb), improved palatability or flavor.

### BRIEF DESCRIPTION OF THE DRAWINGS

[0034] The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

[0035] Figure 1 is a block diagram of a computer system.

[0036] Figure 2 is a flow diagram illustrating one embodiment of a process for comparing a new nucleotide or protein sequence with a database of sequences in order to determine the homology levels between the new sequence and the sequences in the database.

[0037] Figure 3 is a flow diagram illustrating one embodiment of a process in a computer for determining whether two sequences are homologous.

[0038] Figure 4 is a flow diagram illustrating one embodiment of an identifier process 300 for detecting the presence of a feature in a sequence.

[0039] Figure 5 is a graph showing the Residual activity of various amylases following heating to 90°C for 10 min in Example 1.

[0040] Figure 6 is a graph showing the net percent starch removed versus enzyme concentration in ADW wash test with bleach and chelators.

[0041] Figure 7 is a graph showing the activity of parental amylases at pH 8, 40°C in ADW formulation at 55°C.

[0042] Figure 8 is a graph of data regarding the H<sub>2</sub>O<sub>2</sub> tolerance of the novel enzymes in Example 4.

[0043] Figure 9 is a graph of the pH and temperature data for a selection of the amylases characterized. Figure 9a shows the data at pH 8 and 40°C and Figure 9b shows the data at pH 10 and 50°C.

[0044] Figure 10 sets forth the sequences to be used in reassembly experiments with the enzymes.

[0045] Figure 11 illustrates a sample Standard Curve of the assay of Example 5.

[0046] Figure 12 illustrates the pH rate profiles for SEQ ID NO.: 127, which has a neutral optimum pH and SEQ ID NO.: 211, which has an optimum around pH 10.

[0047] Figure 13 shows the stability of Diversa amylases vs. a commercial enzyme, as discussed in Example 2.

[0048] Figure 14 shows the sequence alignments of hypothermophilic  $\alpha$ -amylases, as set forth in Example 8. Figure 14a shows an alignment of amylase sequences. SEQ ID NO.: 81= an environmental clone; pyro = *Pyrococcus sp.* (strain:KOD1), Tachibana, Y., Mendez, L., Takagi, M. and Imanaka, T., J Ferment. Bioeng. 82:224-232, 1996; pyro2 = *Pyrococcus furiosus*, Appl. Environ. Microbiol. 63 (9):3569-3576, 1997; Thermo = *Thermococcus sp.*; Thermo2 = *Thermococcus hydrothermalis*, Leveque, E. et al. Patent: France 98.05655 05-MAY-1998, unpublished. Figure 14b shows the amino acid sequence alignment of identified sequences: SEQ ID NO.: 81; pyro; SEQ ID NO.: 75; SEQ ID NO.: 77; SEQ ID NO.: 83; SEQ ID NO.: 85; thermo2; SEQ ID NO.: 79 ; thermo ; pyro2 ; clone A; thermo3. Figure 14c shows the nucleic acid sequence alignment corresponding to the polypeptide sequence of Figures 5 and 6. SEQ ID NO.: 81; SEQ ID NO.: 75; SEQ ID NO.: 77; SEQ ID NO.: 83; SEQ ID NO.: 85; SEQ ID NO.: 79; clone A; and SEQ ID NO.: 73.

[0049] Figure 15 is a neighbor-joining tree for *Thermococcales*.

[0050] Figure 16 is the sequences of the invention.

#### DETAILED DESCRIPTION OF THE INVENTION

[0051] The present invention relates to amylases and polynucleotides encoding them. As used herein, the term "amylase" encompasses enzymes having alpha amylase activity, for example, alpha amylases capable of hydrolyzing internal  $\alpha$ -1,4-glucan links in polysaccharides, including amylase enzymes capable of hydrolyzing starch to sugars at alkaline pHs or at acidic pHs. Amylases of the invention are particularly useful in corn-wet milling processes, detergents, baking processes, beverages and in oilfields (fuel ethanol). Amylases are also useful in textile desizing, brewing processes, starch modification in the paper and pulp industry and other processes described in the art.

[0052] The polynucleotides of the invention have been identified as encoding polypeptides having alpha amylase or alkaline amylase activity. Alkaline amylases of the invention may include, but are not limited to: SEQ ID NO.: 115, SEQ ID NO.:207, SEQ ID NO.: 139, SEQ ID NO.:127, SEQ ID NO.: 137, SEQ ID NO.:113, SEQ ID NO.:205, SEQ ID NO.: 179, SEQ ID NO.: 151, SEQ ID NO.: 187, SEQ ID NO.:97, SEQ ID NO.: 153, SEQ ID NO.: 69, SEQ ID NO.: 135, SEQ ID NO.: 189, SEQ ID NO.: 119, SEQ ID NO: 209 and SEQ ID NO: 211.

[0053] Alterations in properties which may be achieved in variants of the invention are alterations in, e.g., substrate specificity, substrate binding, substrate cleavage pattern, thermal stability, pH/activity profile, pH/stability profile [such as increased stability at low (e.g. pH<6, in particular pH<5) or high (e.g. pH>9) pH values], stability towards oxidation, Ca<sup>2+</sup> dependency, specific activity, and other properties of interest. For instance, the alteration may result in a variant which, as compared to the parent amylase, has a reduced Ca<sup>2+</sup> dependency and/or an altered pH/activity profile.

[0054] The present invention relates to alpha amylases and polynucleotides encoding them. As used herein, the term "alpha amylase" encompasses enzymes having alpha amylase activity, for example, enzymes capable of hydrolyzing starch to sugars. Unlike many known amylases, the amylases of the invention may not be calcium-dependent enzymes.

[0055] It is highly desirable to be able to decrease the Ca<sup>2+</sup> dependency of an alpha amylase. Accordingly, one aspect of the invention provides an amylase enzyme that has a decreased Ca<sup>2+</sup> dependency as compared to commercial or parent amylases. Decreased Ca<sup>2+</sup> dependency will in general have the functional consequence that the variant exhibits a satisfactory amylolytic activity in the presence of a lower concentration of calcium ion in the extraneous medium than is necessary for a commercial or parent enzyme. It will further often have the consequence that the variant is less sensitive to calcium ion-depleting conditions such as those obtained in media containing calcium-complexing agents (such as certain detergent builders).

[0056] "Liquefaction" or "liquefy" means a process by which starch is converted to shorter chain and less viscous dextrins. Generally, this process involves gelatinization of starch simultaneously with or followed by the addition of alpha amylase. In commercial

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processes, it is preferred that the granular starch is derived from a source comprising corn, wheat, milo, sorghum, rye or bulgher. However, the present invention applies to any grain starch source which is useful in liquefaction, e.g., any other grain or vegetable source known to produce starch suitable for liquefaction.

[0057] "Granular starch" or "starch granules" means a water-insoluble component of edible grains which remains after removal of the hull, fiber, protein, fat, germ, and solubles through the steeping, mechanical cracking, separations, screening, countercurrent rinsing and centrifugation steps typical of the grain wet-milling process. Granular starch comprises intact starch granules containing, almost exclusively, packed starch molecules (i.e., amylopectin and amylose). In corn, the granular starch component comprises about 99% starch; the remaining 1% being comprised of protein, fat, ash, fiber and trace components tightly associated with the granules. The packing structure of granular starch severely retards the ability of .alpha.-amylase to hydrolyze starch. Gelatinization of the starch is utilized to disrupt the granules to form a soluble starch solution and facilitate enzymatic hydrolysis.

[0058] "Starch solution" means the water soluble gelatinized starch which results from heating granular starch. Upon heating of the granules to above about 72 degrees C, granular starch dissociates to form an aqueous mixture of loose starch molecules. This mixture comprising, for example, about 75% amylopectin and 25% amylose in yellow dent corn forms a viscous solution in water. In commercial processes to form glucose or fructose, it is the starch solution which is liquefied to form a soluble dextrin solution. "alpha amylase" means an enzymatic activity which cleaves or hydrolyzes the alpha (1-4) glycosidic bond, e.g., that in starch, amylopectin or amylose polymers. Suitable alpha amylases are the naturally occurring alpha amylases as well as recombinant or mutant amylases which are useful in liquefaction of starch. Techniques for producing variant amylases having activity at a pH or temperature, for example, that is different from the wild-type amylase, are included herein.

[0059] The temperature range of the liquefaction is generally any liquefaction temperature which is known to be effective in liquefying starch. Preferably, the temperature of the starch is between about 80 degrees C to about 115 degrees C, more preferably from



about 100 degrees C to about 110 degrees C, and most preferably from about 105 degrees C to about 108 degrees C.

[0060] In one embodiment, the signal sequences of the invention are identified following identification of novel amylase polypeptides. The pathways by which proteins are sorted and transported to their proper cellular location are often referred to as protein targeting pathways. One of the most important elements in all of these targeting systems is a short amino acid sequence at the amino terminus of a newly synthesized polypeptide called the signal sequence. This signal sequence directs a protein to its appropriate location in the cell and is removed during transport or when the protein reaches its final destination. Most lysosomal, membrane, or secreted proteins have an amino-terminal signal sequence that marks them for translocation into the lumen of the endoplasmic reticulum. More than 100 signal sequences for proteins in this group have been determined. The sequences vary in length from 13 to 36 amino acid residues. Various methods of recognition of signal sequences are known to those of skill in the art. In one embodiment, the peptides are identified by a method referred to as SignalP. SignalP uses a combined neural network which recognizes both signal peptides and their cleavage sites. (Nielsen, H., Engelbrecht, J., Brunak, S., von Heijne, G., "Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites." Protein Engineering, vol. 10, no. 1, p. 1-6 (1997), hereby incorporated by reference.) It should be understood that some of the amylases of the invention may not have signal sequences. It may be desirable to include a nucleic acid sequence encoding a signal sequence from one amylase operably linked to a nucleic acid sequence of a different amylase or, optionally, a signal sequence from a non-amylase protein may be desired. Table 3 shows signal sequences of the invention.

[0061] The phrases "nucleic acid" or "nucleic acid sequence" as used herein refer to an oligonucleotide, nucleotide, polynucleotide, or to a fragment of any of these, to DNA or RNA of genomic or synthetic origin which may be single-stranded or double-stranded and may represent a sense or antisense strand, to peptide nucleic acid (PNA), or to any DNA-like or RNA-like material, natural or synthetic in origin.

[0062] A "coding sequence of" or a "nucleotide sequence encoding" a particular polypeptide or protein, is a nucleic acid sequence which is transcribed and translated into a polypeptide or protein when placed under the control of appropriate regulatory sequences.

[0063] The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as, where applicable, intervening sequences (introns) between individual coding segments (exons).

[0064] "Amino acid" or "amino acid sequence" as used herein refer to an oligopeptide, peptide, polypeptide, or protein sequence, or to a fragment, portion, or subunit of any of these, and to naturally occurring or synthetic molecules.

[0065] The term "polypeptide" as used herein, refers to amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres, and may contain modified amino acids other than the 20 gene-encoded amino acids. The polypeptides may be modified by either natural processes, such as post-translational processing, or by chemical modification techniques which are well known in the art. Modifications can occur anywhere in the polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also a given polypeptide may have many types of modifications. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of a phosphatidylinositol, cross-linking cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, pergylation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, and transfer-RNA mediated addition of amino acids to protein such as arginylation. (See Creighton, T.E., *Proteins – Structure and Molecular Properties* 2nd Ed., W.H. Freeman and Company, New York (1993); *Posttranslational Covalent Modification of Proteins*, B.C. Johnson, Ed., Academic Press, New York, pp. 1-12 (1983)).

[0066] As used herein, the term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

[0067] As used herein, the term "purified" does not require absolute purity; rather, it is intended as a relative definition. Individual nucleic acids obtained from a library have been conventionally purified to electrophoretic homogeneity. The sequences obtained from these clones could not be obtained directly either from the library or from total human DNA. The purified nucleic acids of the invention have been purified from the remainder of the genomic DNA in the organism by at least 10<sup>4</sup>-10<sup>6</sup> fold. However, the term "purified" also includes nucleic acids which have been purified from the remainder of the genomic DNA or from other sequences in a library or other environment by at least one order of magnitude, typically two or three orders, and more typically four or five orders of magnitude.

[0068] As used herein, the term "recombinant" means that the nucleic acid is adjacent to a "backbone" nucleic acid to which it is not adjacent in its natural environment. Additionally, to be "enriched" the nucleic acids will represent 5% or more of the number of nucleic acid inserts in a population of nucleic acid backbone molecules. Backbone molecules according to the invention include nucleic acids such as expression vectors, self-replicating nucleic acids, viruses, integrating nucleic acids, and other vectors or nucleic acids used to maintain or manipulate a nucleic acid insert of interest. Typically, the enriched nucleic acids represent 15% or more of the number of nucleic acid inserts in the population of recombinant backbone molecules. More typically, the enriched nucleic acids represent 50% or more of the number of nucleic acid inserts in the population of recombinant backbone molecules. In a one embodiment, the enriched nucleic acids represent 90% or more of the number of nucleic acid inserts in the population of recombinant backbone molecules.

[0069] "Recombinant" polypeptides or proteins refer to polypeptides or proteins produced by recombinant DNA techniques; i.e., produced from cells transformed by an

exogenous DNA construct encoding the desired polypeptide or protein. "Synthetic" polypeptides or protein are those prepared by chemical synthesis. Solid-phase chemical peptide synthesis methods can also be used to synthesize the polypeptide or fragments of the invention. Such method have been known in the art since the early 1960's (Merrifield, R. B., J. Am. Chem. Soc., 85:2149-2154, 1963) (See also Stewart, J. M. and Young, J. D., Solid Phase Peptide Synthesis, 2nd Ed., Pierce Chemical Co., Rockford, Ill., pp. 11-12)) and have recently been employed in commercially available laboratory peptide design and synthesis kits (Cambridge Research Biochemicals). Such commercially available laboratory kits have generally utilized the teachings of H. M. Geysen et al, Proc. Natl. Acad. Sci., USA, 81:3998 (1984) and provide for synthesizing peptides upon the tips of a multitude of "rods" or "pins" all of which are connected to a single plate. When such a system is utilized, a plate of rods or pins is inverted and inserted into a second plate of corresponding wells or reservoirs, which contain solutions for attaching or anchoring an appropriate amino acid to the pin's or rod's tips. By repeating such a process step, i.e., inverting and inserting the rod's and pin's tips into appropriate solutions, amino acids are built into desired peptides. In addition, a number of available Fmoc peptide synthesis systems are available. For example, assembly of a polypeptide or fragment can be carried out on a solid support using an Applied Biosystems, Inc. Model 431A automated peptide synthesizer. Such equipment provides ready access to the peptides of the invention, either by direct synthesis or by synthesis of a series of fragments that can be coupled using other known techniques.

[0070] A promoter sequence is "operably linked to" a coding sequence when RNA polymerase which initiates transcription at the promoter will transcribe the coding sequence into mRNA.

[0071] "Plasmids" are designated by a lower case "p" preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described herein are known in the art and will be apparent to the ordinarily skilled artisan.

[0072] "Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used

herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1 µg of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 µl of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 µg of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion, gel electrophoresis may be performed to isolate the desired fragment.

[0073] "Oligonucleotide" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

[0074] The phrase "substantially identical" in the context of two nucleic acids or polypeptides, refers to two or more sequences that have at least 50%, 60%, 70%, 80%, and in some aspects 90-95% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using one of the known sequence comparison algorithms or by visual inspection. Typically, the substantial identity exists over a region of at least about 100 residues, and most commonly the sequences are substantially identical over at least about 150-200 residues. In some embodiments, the sequences are substantially identical over the entire length of the coding regions.

[0075] Additionally a "substantially identical" amino acid sequence is a sequence that differs from a reference sequence by one or more conservative or non-conservative amino acid substitutions, deletions, or insertions, particularly when such a substitution occurs at a site that is not the active site of the molecule, and provided that the polypeptide essentially retains its functional properties. A conservative amino acid substitution, for example, substitutes one amino acid for another of the same class (e.g., substitution of one hydrophobic amino acid, such as isoleucine, valine, leucine, or methionine, for another, or substitution of one polar amino acid for another, such as substitution of arginine for lysine, glutamic acid for

aspartic acid or glutamine for asparagine). One or more amino acids can be deleted, for example, from an alpha amylase polypeptide, resulting in modification of the structure of the polypeptide, without significantly altering its biological activity. For example, amino- or carboxyl-terminal amino acids that are not required for alpha amylase biological activity can be removed. Modified polypeptide sequences of the invention can be assayed for alpha amylase biological activity by any number of methods, including contacting the modified polypeptide sequence with an alpha amylase substrate and determining whether the modified polypeptide decreases the amount of specific substrate in the assay or increases the bioproducts of the enzymatic reaction of a functional alpha amylase polypeptide with the substrate.

[0076] "Fragments" as used herein are a portion of a naturally occurring protein which can exist in at least two different conformations. Fragments can have the same or substantially the same amino acid sequence as the naturally occurring protein. "Substantially the same" means that an amino acid sequence is largely, but not entirely, the same, but retains at least one functional activity of the sequence to which it is related. In general two amino acid sequences are "substantially the same" or "substantially homologous" if they are at least about 85% identical. Fragments which have different three dimensional structures as the naturally occurring protein are also included. An example of this, is a "pro-form" molecule, such as a low activity proprotein that can be modified by cleavage to produce a mature enzyme with significantly higher activity.

[0077] "Hybridization" refers to the process by which a nucleic acid strand joins with a complementary strand through base pairing. Hybridization reactions can be sensitive and selective so that a particular sequence of interest can be identified even in samples in which it is present at low concentrations. Suitably stringent conditions can be defined by, for example, the concentrations of salt or formamide in the prehybridization and hybridization solutions, or by the hybridization temperature, and are well known in the art. In particular, stringency can be increased by reducing the concentration of salt, increasing the concentration of formamide, or raising the hybridization temperature.

[0078] For example, hybridization under high stringency conditions could occur in about 50% formamide at about 37°C to 42°C. Hybridization could occur under reduced

stringency conditions in about 35% to 25% formamide at about 30°C to 35°C. In particular, hybridization could occur under high stringency conditions at 42°C in 50% formamide, 5X SSPE, 0.3% SDS, and 200 n/ml sheared and denatured salmon sperm DNA. Hybridization could occur under reduced stringency conditions as described above, but in 35% formamide at a reduced temperature of 35°C. The temperature range corresponding to a particular level of stringency can be further narrowed by calculating the purine to pyrimidine ratio of the nucleic acid of interest and adjusting the temperature accordingly. Variations on the above ranges and conditions are well known in the art.

[0079] The term "variant" refers to polynucleotides or polypeptides of the invention modified at one or more base pairs, codons, introns, exons, or amino acid residues (respectively) yet still retain the biological activity of an alpha amylase of the invention. Variants can be produced by any number of means included methods such as, for example, error-prone PCR, shuffling, oligonucleotide-directed mutagenesis, assembly PCR, sexual PCR mutagenesis, *in vivo* mutagenesis, cassette mutagenesis, recursive ensemble mutagenesis, exponential ensemble mutagenesis, site-specific mutagenesis, gene reassembly, GSSM and any combination thereof. Techniques for producing variant amylases having activity at a pH or temperature, for example, that is different from the wild-type amylase, are included herein.

[0080] Enzymes are highly selective catalysts. Their hallmark is the ability to catalyze reactions with exquisite stereo-, regio-, and chemo- selectivities that are unparalleled in conventional synthetic chemistry. Moreover, enzymes are remarkably versatile. They can be tailored to function in organic solvents, operate at extreme pHs (for example, high pHs and low pHs) extreme temperatures (for example, high temperatures and low temperatures), extreme salinity levels (for example, high salinity and low salinity), and catalyze reactions with compounds that are structurally unrelated to their natural, physiological substrates.

[0081] Enzymes are reactive toward a wide range of natural and unnatural substrates, thus enabling the modification of virtually any organic lead compound. Moreover, unlike traditional chemical catalysts, enzymes are highly enantio- and regio-selective. The high degree of functional group specificity exhibited by enzymes enables one to keep track of each reaction in a synthetic sequence leading to a new active compound. Enzymes are also capable

of catalyzing many diverse reactions unrelated to their physiological function in nature. For example, peroxidases catalyze the oxidation of phenols by hydrogen peroxide. Peroxidases can also catalyze hydroxylation reactions that are not related to the native function of the enzyme. Other examples are proteases which catalyze the breakdown of polypeptides. In organic solution some proteases can also acylate sugars, a function unrelated to the native function of these enzymes.

[0082] In one aspect, the invention includes a method for liquefying a starch containing composition comprising contacting the starch with a polypeptide of the invention (e.g., a purified polypeptide selected from polypeptides having an amino acid sequence selected from the group consisting of: Group B amino acid sequences; variants having at least about 50% homology to at least one of Group B amino acid sequences, over a region of at least about 100 residues, as determined by analysis with a sequence comparison algorithm or by visual inspection; sequences complementary to any one of Group B amino acid sequences; and sequences complementary to variants having at least about 50% homology to any one of Group B amino acid sequences over a region of at least about 100 residues, as determined by analysis with a sequence comparison algorithm or by visual inspection; and polypeptides having at least 10 consecutive amino acids of a polypeptide having a sequence selected from the group consisting of Group B amino acid sequences). In one preferred embodiment, the polypeptide is set forth in Group B amino acid sequences. The starch may be from a material selected from rice, germinated rice, corn, barley, wheat, legumes and sweet potato. A glucose syrup produced by the method of the invention is included herein. Such a syrup can be a maltose syrup, a glucose syrup, or a combination thereof. In particular, the syrups produced using the amylases of the invention there is a higher level of DP2 fraction and a higher level of DP3 (maltotriose and/or panose) and less of the greater than DP7 fragments as compared to the syrups produced by commercial enzymes. This is consistent with the liquefaction profile since less of the large fragments are in the invention liquefied syrups.

[0083] The invention also provides a method for removing starch containing stains from a material comprising contacting the material with a polypeptide of the invention. In one aspect, the invention provides a method for washing an object comprising contacting the object with a polypeptide of the invention under conditions sufficient for washing. A



polypeptide of the invention may be included as a detergent additive for example. The invention also includes a method for textile desizing comprising contacting the textile with a polypeptide of the invention under conditions sufficient for desizing.

[0084] The invention also provides a method of reducing the staling of bakery products comprising addition of a polypeptide of the invention to the bakery product, prior to baking.

[0085] The invention also provides a method for the treatment of lignocellulosic fibers, wherein the fibers are treated with a polypeptide of the invention, in an amount which is efficient for improving the fiber properties. The invention includes a for enzymatic deinking of recycled paper pulp, wherein the polypeptide is applied in an amount which is efficient for effective deinking of the fiber surface.

[0086] Any of the methods described herein include the possibility of the addition of a second alpha amylase or a beta amylase or a combination thereof. Commercial amylases or other enzymes suitable for use in combination with an enzyme of the invention are known to those of skill in the art.

[0087] The invention also includes a method of increasing the flow of production fluids from a subterranean formation by removing a viscous, starch-containing, damaging fluid formed during production operations and found within the subterranean formation which surrounds a completed well bore comprising allowing production fluids to flow from the well bore; reducing the flow of production fluids from the formation below expected flow rates; formulating an enzyme treatment by blending together an aqueous fluid and a polypeptide of the invention; pumping the enzyme treatment to a desired location within the well bore; allowing the enzyme treatment to degrade the viscous, starch-containing, damaging fluid, whereby the fluid can be removed from the subterranean formation to the well surface; and wherein the enzyme treatment is effective to attack the alpha glucosidic linkages in the starch-containing fluid.

[0088] The present invention exploits the unique catalytic properties of enzymes. Whereas the use of biocatalysts (i.e., purified or crude enzymes, non-living or living cells) in chemical transformations normally requires the identification of a particular biocatalyst that

reacts with a specific starting compound, the present invention uses selected biocatalysts and reaction conditions that are specific for functional groups that are present in many starting compounds.

[0089] Each biocatalyst is specific for one functional group, or several related functional groups, and can react with many starting compounds containing this functional group.

[0090] The biocatalytic reactions produce a population of derivatives from a single starting compound. These derivatives can be subjected to another round of biocatalytic reactions to produce a second population of derivative compounds. Thousands of variations of the original compound can be produced with each iteration of biocatalytic derivatization.

[0091] Enzymes react at specific sites of a starting compound without affecting the rest of the molecule, a process which is very difficult to achieve using traditional chemical methods. This high degree of biocatalytic specificity provides the means to identify a single active compound within the library. The library is characterized by the series of biocatalytic reactions used to produce it, a so-called "biosynthetic history". Screening the library for biological activities and tracing the biosynthetic history identifies the specific reaction sequence producing the active compound. The reaction sequence is repeated and the structure of the synthesized compound determined. This mode of identification, unlike other synthesis and screening approaches, does not require immobilization technologies, and compounds can be synthesized and tested free in solution using virtually any type of screening assay. It is important to note, that the high degree of specificity of enzyme reactions on functional groups allows for the "tracking" of specific enzymatic reactions that make up the biocatalytically produced library.

[0092] There are many advantages to screening lambda phage libraries for expression-based discovery of amylases. These include improved detection of toxic clones; improved access to substrate; reduced need for engineering a host; by-passing the potential for any bias resulting from mass excision of the library; and faster growth at low clone densities. Additionally, there are advantages to screening lambda phage libraries in liquid phase over solid phase. These include: greater flexibility in assay conditions; additional substrate flexibility; higher sensitivity for weak clones; and ease of automation.

**[0093]** Many of the procedural steps are performed using robotic automation enabling the execution of many thousands of biocatalytic reactions and screening assays per day as well as ensuring a high level of accuracy and reproducibility. As a result, a library of derivative compounds can be produced in a matter of weeks which would take years to produce using current chemical methods. (For further teachings on modification of molecules, including small molecules, see PCT/US94/09174, herein incorporated by reference in its entirety).

**[0094]** In one aspect, the present invention provides a non-stochastic method termed synthetic gene reassembly, that is somewhat related to stochastic shuffling, save that the nucleic acid building blocks are not shuffled or concatenated or chimerized randomly, but rather are assembled non-stochastically.

[0095] The synthetic gene reassembly method does not depend on the presence of a high level of homology between polynucleotides to be shuffled. The invention can be used to non-stochastically generate libraries (or sets) of progeny molecules comprised of over  $10^{100}$  different chimeras. Conceivably, synthetic gene reassembly can even be used to generate libraries comprised of over  $10^{1000}$  different progeny chimeras. Thus, in one aspect, the invention provides a non-stochastic method of producing a set of finalized chimeric nucleic acid molecules having an overall assembly order that is chosen by design, which method is comprised of the steps of generating by design a plurality of specific nucleic acid building blocks having serviceable mutually compatible ligatable ends, and assembling these nucleic acid building blocks, such that a designed overall assembly order is achieved.

**[0096]** The mutually compatible ligatable ends of the nucleic acid building blocks to be assembled are considered to be “serviceable” for this type of ordered assembly if they enable the building blocks to be coupled in predetermined orders. Thus, in one aspect, the overall assembly order in which the nucleic acid building blocks can be coupled is specified by the design of the ligatable ends and, if more than one assembly step is to be used, then the overall assembly order in which the nucleic acid building blocks can be coupled is also specified by the sequential order of the assembly step(s). In a one embodiment of the invention, the annealed building pieces are treated with an enzyme, such as a ligase (e.g., T4 DNA ligase) to achieve covalent bonding of the building pieces.

[0097] In a another embodiment, the design of nucleic acid building blocks is obtained upon analysis of the sequences of a set of progenitor nucleic acid templates that serve as a basis for producing a progeny set of finalized chimeric nucleic acid molecules. These progenitor nucleic acid templates thus serve as a source of sequence information that aids in the design of the nucleic acid building blocks that are to be mutagenized, e.g., chimerized or shuffled.

[0098] In one exemplification, the invention provides for the chimerization of a family of related genes and their encoded family of related products. In a particular exemplification, the encoded products are enzymes. The amylases of the present invention, for example, alpha amylases or alkaline amylases, can be mutagenized in accordance with the methods described herein.

[0099] Thus according to one aspect of the invention, the sequences of a plurality of progenitor nucleic acid templates (e.g., polynucleotides of Group A nucleic acid sequences) are aligned in order to select one or more demarcation points, which demarcation points can be located at an area of homology. The demarcation points can be used to delineate the boundaries of nucleic acid building blocks to be generated. Thus, the demarcation points identified and selected in the progenitor molecules serve as potential chimerization points in the assembly of the progeny molecules.

[0100] Typically a serviceable demarcation point is an area of homology (comprised of at least one homologous nucleotide base) shared by at least two progenitor templates, but the demarcation point can be an area of homology that is shared by at least half of the progenitor templates, at least two thirds of the progenitor templates, at least three fourths of the progenitor templates, and preferably at almost all of the progenitor templates. Even more preferably still a serviceable demarcation point is an area of homology that is shared by all of the progenitor templates.

[0101] In a one embodiment, the gene reassembly process is performed exhaustively in order to generate an exhaustive library. In other words, all possible ordered combinations of the nucleic acid building blocks are represented in the set of finalized chimeric nucleic acid molecules. At the same time, the assembly order (i.e. the order of assembly of each building block in the 5' to 3' sequence of each finalized chimeric nucleic acid) in each

combination is by design (or non-stochastic). Because of the non-stochastic nature of the method, the possibility of unwanted side products is greatly reduced.

[0102] In another embodiment, the method provides that the gene reassembly process is performed systematically, for example to generate a systematically compartmentalized library, with compartments that can be screened systematically, e.g., one by one. In other words the invention provides that, through the selective and judicious use of specific nucleic acid building blocks, coupled with the selective and judicious use of sequentially stepped assembly reactions, an experimental design can be achieved where specific sets of progeny products are made in each of several reaction vessels. This allows a systematic examination and screening procedure to be performed. Thus, it allows a potentially very large number of progeny molecules to be examined systematically in smaller groups.

[0103] Because of its ability to perform chimerizations in a manner that is highly flexible yet exhaustive and systematic as well, particularly when there is a low level of homology among the progenitor molecules, the instant invention provides for the generation of a library (or set) comprised of a large number of progeny molecules. Because of the non-stochastic nature of the instant gene reassembly invention, the progeny molecules generated preferably comprise a library of finalized chimeric nucleic acid molecules having an overall assembly order that is chosen by design. In a particularly embodiment, such a generated library is comprised of greater than 103 to greater than 101000 different progeny molecular species.

[0104] In one aspect, a set of finalized chimeric nucleic acid molecules, produced as described is comprised of a polynucleotide encoding a polypeptide. According to one embodiment, this polynucleotide is a gene, which may be a man-made gene. According to another embodiment, this polynucleotide is a gene pathway, which may be a man-made gene pathway. The invention provides that one or more man-made genes generated by the invention may be incorporated into a man-made gene pathway, such as pathway operable in a eukaryotic organism (including a plant).

[0105] In another exemplification, the synthetic nature of the step in which the building blocks are generated allows the design and introduction of nucleotides (e.g., one or more nucleotides, which may be, for example, codons or introns or regulatory sequences) that

can later be optionally removed in an *in vitro* process (e.g., by mutagenesis) or in an *in vivo* process (e.g., by utilizing the gene splicing ability of a host organism). It is appreciated that in many instances the introduction of these nucleotides may also be desirable for many other reasons in addition to the potential benefit of creating a serviceable demarcation point.

[0106] Thus, according to another embodiment, the invention provides that a nucleic acid building block can be used to introduce an intron. Thus, the invention provides that functional introns may be introduced into a man-made gene of the invention. The invention also provides that functional introns may be introduced into a man-made gene pathway of the invention. Accordingly, the invention provides for the generation of a chimeric polynucleotide that is a man-made gene containing one (or more) artificially introduced intron(s).

[0107] Accordingly, the invention also provides for the generation of a chimeric polynucleotide that is a man-made gene pathway containing one (or more) artificially introduced intron(s). Preferably, the artificially introduced intron(s) are functional in one or more host cells for gene splicing much in the way that naturally-occurring introns serve functionally in gene splicing. The invention provides a process of producing man-made intron-containing polynucleotides to be introduced into host organisms for recombination and/or splicing.

[0108] A man-made gene produced using the invention can also serve as a substrate for recombination with another nucleic acid. Likewise, a man-made gene pathway produced using the invention can also serve as a substrate for recombination with another nucleic acid. In a preferred instance, the recombination is facilitated by, or occurs at, areas of homology between the man-made, intron-containing gene and a nucleic acid, which serves as a recombination partner. In a particularly preferred instance, the recombination partner may also be a nucleic acid generated by the invention, including a man-made gene or a man-made gene pathway. Recombination may be facilitated by or may occur at areas of homology that exist at the one (or more) artificially introduced intron(s) in the man-made gene.

[0109] The synthetic gene reassembly method of the invention utilizes a plurality of nucleic acid building blocks, each of which preferably has two ligatable ends. The two ligatable ends on each nucleic acid building block may be two blunt ends (i.e. each having an

overhang of zero nucleotides), or preferably one blunt end and one overhang, or more preferably still two overhangs.

[0110] A useful overhang for this purpose may be a 3' overhang or a 5' overhang. Thus, a nucleic acid building block may have a 3' overhang or alternatively a 5' overhang or alternatively two 3' overhangs or alternatively two 5' overhangs. The overall order in which the nucleic acid building blocks are assembled to form a finalized chimeric nucleic acid molecule is determined by purposeful experimental design and is not random.

[0111] According to one preferred embodiment, a nucleic acid building block is generated by chemical synthesis of two single-stranded nucleic acids (also referred to as single-stranded oligos) and contacting them so as to allow them to anneal to form a double-stranded nucleic acid building block.

[0112] A double-stranded nucleic acid building block can be of variable size. The sizes of these building blocks can be small or large. Preferred sizes for building block range from 1 base pair (not including any overhangs) to 100,000 base pairs (not including any overhangs). Other preferred size ranges are also provided, which have lower limits of from 1 bp to 10,000 bp (including every integer value in between), and upper limits of from 2 bp to 100, 000 bp (including every integer value in between).

[0113] Many methods exist by which a double-stranded nucleic acid building block can be generated that is serviceable for the invention; and these are known in the art and can be readily performed by the skilled artisan.

[0114] According to one embodiment, a double-stranded nucleic acid building block is generated by first generating two single stranded nucleic acids and allowing them to anneal to form a double-stranded nucleic acid building block. The two strands of a double-stranded nucleic acid building block may be complementary at every nucleotide apart from any that form an overhang; thus containing no mismatches, apart from any overhang(s). According to another embodiment, the two strands of a double-stranded nucleic acid building block are complementary at fewer than every nucleotide apart from any that form an overhang. Thus, according to this embodiment, a double-stranded nucleic acid building block can be used to introduce codon degeneracy. Preferably the codon degeneracy is introduced using the site-

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saturation mutagenesis described herein, using one or more N,N,G/T cassettes or alternatively using one or more N,N,N cassettes.

[0115] The *in vivo* recombination method of the invention can be performed blindly on a pool of unknown hybrids or alleles of a specific polynucleotide or sequence. However, it is not necessary to know the actual DNA or RNA sequence of the specific polynucleotide.

[0116] The approach of using recombination within a mixed population of genes can be useful for the generation of any useful proteins, for example, interleukin I, antibodies, tPA and growth hormone. This approach may be used to generate proteins having altered specificity or activity. The approach may also be useful for the generation of hybrid nucleic acid sequences, for example, promoter regions, introns, exons, enhancer sequences, 3' untranslated regions or 5' untranslated regions of genes. Thus this approach may be used to generate genes having increased rates of expression. This approach may also be useful in the study of repetitive DNA sequences. Finally, this approach may be useful to mutate ribozymes or aptamers.

[0117] In one aspect the invention described herein is directed to the use of repeated cycles of reductive reassortment, recombination and selection which allow for the directed molecular evolution of highly complex linear sequences, such as DNA, RNA or proteins thorough recombination.

[0118] *In vivo* shuffling of molecules is useful in providing variants and can be performed utilizing the natural property of cells to recombine multimers. While recombination *in vivo* has provided the major natural route to molecular diversity, genetic recombination remains a relatively complex process that involves 1) the recognition of homologies; 2) strand cleavage, strand invasion, and metabolic steps leading to the production of recombinant chiasma; and finally 3) the resolution of chiasma into discrete recombined molecules. The formation of the chiasma requires the recognition of homologous sequences.

[0119] In another embodiment, the invention includes a method for producing a hybrid polynucleotide from at least a first polynucleotide and a second polynucleotide. The invention can be used to produce a hybrid polynucleotide by introducing at least a first



polynucleotide and a second polynucleotide which share at least one region of partial sequence homology into a suitable host cell. The regions of partial sequence homology promote processes which result in sequence reorganization producing a hybrid polynucleotide. The term "hybrid polynucleotide", as used herein, is any nucleotide sequence which results from the method of the present invention and contains sequence from at least two original polynucleotide sequences. Such hybrid polynucleotides can result from intermolecular recombination events which promote sequence integration between DNA molecules. In addition, such hybrid polynucleotides can result from intramolecular reductive reassortment processes which utilize repeated sequences to alter a nucleotide sequence within a DNA molecule.

[0120] The invention provides a means for generating hybrid polynucleotides which may encode biologically active hybrid polypeptides (e.g., hybrid alpha amylases). In one aspect, the original polynucleotides encode biologically active polypeptides. The method of the invention produces new hybrid polypeptides by utilizing cellular processes which integrate the sequence of the original polynucleotides such that the resulting hybrid polynucleotide encodes a polypeptide demonstrating activities derived from the original biologically active polypeptides. For example, the original polynucleotides may encode a particular enzyme from different microorganisms. An enzyme encoded by a first polynucleotide from one organism or variant may, for example, function effectively under a particular environmental condition, e.g. high salinity. An enzyme encoded by a second polynucleotide from a different organism or variant may function effectively under a different environmental condition, such as extremely high temperatures. A hybrid polynucleotide containing sequences from the first and second original polynucleotides may encode an enzyme which exhibits characteristics of both enzymes encoded by the original polynucleotides. Thus, the enzyme encoded by the hybrid polynucleotide may function effectively under environmental conditions shared by each of the enzymes encoded by the first and second polynucleotides, e.g., high salinity and extreme temperatures.

[0121] Enzymes encoded by the polynucleotides of the invention include, but are not limited to, hydrolases, such as alpha amylases and alkaline amylases. A hybrid polypeptide resulting from the method of the invention may exhibit specialized enzyme activity not displayed in the original enzymes. For example, following recombination and/or reductive

reassortment of polynucleotides encoding hydrolase activities, the resulting hybrid polypeptide encoded by a hybrid polynucleotide can be screened for specialized hydrolase activities obtained from each of the original enzymes, i.e. the type of bond on which the hydrolase acts and the temperature at which the hydrolase functions. Thus, for example, the hydrolase may be screened to ascertain those chemical functionalities which distinguish the hybrid hydrolase from the original hydrolases, such as: (a) amide (peptide bonds), i.e., proteases; (b) ester bonds, i.e., amylases and lipases; (c) acetals, i.e., glycosidases and, for example, the temperature, pH or salt concentration at which the hybrid polypeptide functions.

[0122] Sources of the original polynucleotides may be isolated from individual organisms ("isolates"), collections of organisms that have been grown in defined media ("enrichment cultures"), or, uncultivated organisms ("environmental samples"). The use of a culture-independent approach to derive polynucleotides encoding novel bioactivities from environmental samples is most preferable since it allows one to access untapped resources of biodiversity.

[0123] "Environmental libraries" are generated from environmental samples and represent the collective genomes of naturally occurring organisms archived in cloning vectors that can be propagated in suitable prokaryotic hosts. Because the cloned DNA is initially extracted directly from environmental samples, the libraries are not limited to the small fraction of prokaryotes that can be grown in pure culture. Additionally, a normalization of the environmental DNA present in these samples could allow more equal representation of the DNA from all of the species present in the original sample. This can dramatically increase the efficiency of finding interesting genes from minor constituents of the sample which may be under-represented by several orders of magnitude compared to the dominant species.

[0124] For example, gene libraries generated from one or more uncultivated microorganisms are screened for an activity of interest. Potential pathways encoding bioactive molecules of interest are first captured in prokaryotic cells in the form of gene expression libraries. Polynucleotides encoding activities of interest are isolated from such libraries and introduced into a host cell. The host cell is grown under conditions which

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promote recombination and/or reductive reassortment creating potentially active biomolecules with novel or enhanced activities.

[0125] The microorganisms from which the polynucleotide may be prepared include prokaryotic microorganisms, such as *Eubacteria* and *Archaeobacteria*, and lower eukaryotic microorganisms such as fungi, some algae and protozoa. Polynucleotides may be isolated from environmental samples in which case the nucleic acid may be recovered without culturing of an organism or recovered from one or more cultured organisms. In one aspect, such microorganisms may be extremophiles, such as hyperthermophiles, psychrophiles, psychrotrophs, halophiles, barophiles and acidophiles. Polynucleotides encoding enzymes isolated from extremophilic microorganisms are particularly preferred. Such enzymes may function at temperatures above 100°C in terrestrial hot springs and deep sea thermal vents, at temperatures below 0°C in arctic waters, in the saturated salt environment of the Dead Sea, at pH values around 0 in coal deposits and geothermal sulfur-rich springs, or at pH values greater than 11 in sewage sludge. For example, several amylases and lipases cloned and expressed from extremophilic organisms show high activity throughout a wide range of temperatures and pHs.

[0126] Of the novel enzymes of the present invention, many have been purified and characterized at pH 8, at both 40°C and 50°C, and pH 10 at both 40°C and 50°C. Of the enzymes found to be purified and characterized at pH 8 and 40°C, was seen to have three times (682 U/mg) the specific activity of a *B. licheniformis* enzyme (228 U/mg). Additionally, another enzyme was seen to have approximately equivalent activity (250U/mg) to the *B. licheniformis* enzyme. At a pH 10 and 50°C, one of the enzymes has a specific activity of 31U/mg and another has a specific activity of 27.5 U/mg, while *B. licheniformis* has a specific activity of 27 U/mg.

[0127] Polynucleotides selected and isolated as hereinabove described are introduced into a suitable host cell. A suitable host cell is any cell which is capable of promoting recombination and/or reductive reassortment. The selected polynucleotides are preferably already in a vector which includes appropriate control sequences. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or preferably, the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction

of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis et al., 1986).

[0128] As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as *E. coli*, *Streptomyces*, *Salmonella typhimurium*; fungal cells, such as yeast; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9; animal cells such as CHO, COS or Bowes melanoma; adenoviruses; and plant cells. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

[0129] With particular references to various mammalian cell culture systems that can be employed to express recombinant protein, examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described in "SV40-transformed simian cells support the replication of early SV40 mutants" (Gluzman, 1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

[0130] Host cells containing the polynucleotides of interest can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan. The clones which are identified as having the specified enzyme activity may then be sequenced to identify the polynucleotide sequence encoding an enzyme having the enhanced activity.

[0131] In another aspect, it is envisioned the method of the present invention can be used to generate novel polynucleotides encoding biochemical pathways from one or more operons or gene clusters or portions thereof. For example, bacteria and many eukaryotes have a coordinated mechanism for regulating genes whose products are involved in related processes. The genes are clustered, in structures referred to as "gene clusters," on a single

chromosome and are transcribed together under the control of a single regulatory sequence, including a single promoter which initiates transcription of the entire cluster. Thus, a gene cluster is a group of adjacent genes that are either identical or related, usually as to their function. An example of a biochemical pathway encoded by gene clusters are polyketides. Polyketides are molecules which are an extremely rich source of bioactivities, including antibiotics (such as tetracyclines and erythromycin), anti-cancer agents (daunomycin), immunosuppressants (FK506 and rapamycin), and veterinary products (monensin). Many polyketides (produced by polyketide synthases) are valuable as therapeutic agents. Polyketide synthases are multifunctional enzymes that catalyze the biosynthesis of an enormous variety of carbon chains differing in length and patterns of functionality and cyclization. Polyketide synthase genes fall into gene clusters and at least one type (designated type I) of polyketide synthases have large size genes and enzymes, complicating genetic manipulation and *in vitro* studies of these genes/proteins.

[0132] Gene cluster DNA can be isolated from different organisms and ligated into vectors, particularly vectors containing expression regulatory sequences which can control and regulate the production of a detectable protein or protein-related array activity from the ligated gene clusters. Use of vectors which have an exceptionally large capacity for exogenous DNA introduction are particularly appropriate for use with such gene clusters and are described by way of example herein to include the f-factor (or fertility factor) of *E. coli*. This f-factor of *E. coli* is a plasmid which affect high-frequency transfer of itself during conjugation and is ideal to achieve and stably propagate large DNA fragments, such as gene clusters from mixed microbial samples. A particularly preferred embodiment is to use cloning vectors, referred to as "fosmids" or bacterial artificial chromosome (BAC) vectors. These are derived from *E. coli* f-factor which is able to stably integrate large segments of genomic DNA. When integrated with DNA from a mixed uncultured environmental sample, this makes it possible to achieve large genomic fragments in the form of a stable "environmental DNA library." Another type of vector for use in the present invention is a cosmid vector. Cosmid vectors were originally designed to clone and propagate large segments of genomic DNA. Cloning into cosmid vectors is described in detail in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press (1989). Once ligated into an appropriate vector, two or more vectors containing different polyketide

synthase gene clusters can be introduced into a suitable host cell. Regions of partial sequence homology shared by the gene clusters will promote processes which result in sequence reorganization resulting in a hybrid gene cluster. The novel hybrid gene cluster can then be screened for enhanced activities not found in the original gene clusters.

[0133] Therefore, in a one embodiment, the invention relates to a method for producing a biologically active hybrid polypeptide and screening such a polypeptide for enhanced activity by:

- [0134] 1) introducing at least a first polynucleotide in operable linkage and a second polynucleotide in operable linkage, said at least first polynucleotide and second polynucleotide sharing at least one region of partial sequence homology, into a suitable host cell;
- [0135] 2) growing the host cell under conditions which promote sequence reorganization resulting in a hybrid polynucleotide in operable linkage;
- [0136] 3) expressing a hybrid polypeptide encoded by the hybrid polynucleotide;
- [0137] 4) screening the hybrid polypeptide under conditions which promote identification of enhanced biological activity; and
- [0138] 5) isolating the a polynucleotide encoding the hybrid polypeptide.

[0139] Methods for screening for various enzyme activities are known to those of skill in the art and are discussed throughout the present specification. Such methods may be employed when isolating the polypeptides and polynucleotides of the invention.

[0140] As representative examples of expression vectors which may be used, there may be mentioned viral particles, baculovirus, phage, plasmids, phagemids, cosmids, fosmids, bacterial artificial chromosomes, viral DNA (e.g., vaccinia, adenovirus, fowl pox virus, pseudorabies and derivatives of SV40), P1-based artificial chromosomes, yeast plasmids, yeast artificial chromosomes, and any other vectors specific for specific hosts of interest (such as bacillus, aspergillus and yeast). Thus, for example, the DNA may be included in any one of a variety of expression vectors for expressing a polypeptide. Such

vectors include chromosomal, nonchromosomal and synthetic DNA sequences. Large numbers of suitable vectors are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example; Bacterial: pQE vectors (Qiagen), pBluescript plasmids, pNH vectors, (lambda-ZAP vectors (Stratagene); ptrc99a, pKK223-3, pDR540, pRIT2T (Pharmacia); Eukaryotic: pXT1, pSG5 (Stratagene), pSVK3, pBPV, pMSG, pSVLSV40 (Pharmacia). However, any other plasmid or other vector may be used so long as they are replicable and viable in the host. Low copy number or high copy number vectors may be employed with the present invention.

[0141] The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct RNA synthesis. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda PR, PL and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression. Promoter regions can be selected from any desired gene using chloramphenicol transferase (CAT) vectors or other vectors with selectable markers. In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E. coli*.

[0142] *In vivo* reassortment is focused on "inter-molecular" processes collectively referred to as "recombination" which in bacteria, is generally viewed as a "RecA-dependent" phenomenon. The invention can rely on recombination processes of a host cell to recombine and re-assort sequences, or the cells' ability to mediate reductive processes to decrease the complexity of quasi-repeated sequences in the cell by deletion. This process of "reductive reassortment" occurs by an "intra-molecular", RecA-independent process.

[0143] Therefore, in another aspect of the invention, novel polynucleotides can be generated by the process of reductive reassortment. The method involves the generation of constructs containing consecutive sequences (original encoding sequences), their insertion

into an appropriate vector, and their subsequent introduction into an appropriate host cell. The reassortment of the individual molecular identities occurs by combinatorial processes between the consecutive sequences in the construct possessing regions of homology, or between quasi-repeated units. The reassortment process recombines and/or reduces the complexity and extent of the repeated sequences, and results in the production of novel molecular species. Various treatments may be applied to enhance the rate of reassortment. These could include treatment with ultra-violet light, or DNA damaging chemicals, and/or the use of host cell lines displaying enhanced levels of "genetic instability". Thus the reassortment process may involve homologous recombination or the natural property of quasi-repeated sequences to direct their own evolution.

[0144] Repeated or "quasi-repeated" sequences play a role in genetic instability. In the present invention, "quasi-repeats" are repeats that are not restricted to their original unit structure. Quasi-repeated units can be presented as an array of sequences in a construct; consecutive units of similar sequences. Once ligated, the junctions between the consecutive sequences become essentially invisible and the quasi-repetitive nature of the resulting construct is now continuous at the molecular level. The deletion process the cell performs to reduce the complexity of the resulting construct operates between the quasi-repeated sequences. The quasi-repeated units provide a practically limitless repertoire of templates upon which slippage events can occur. The constructs containing the quasi-repeats thus effectively provide sufficient molecular elasticity that deletion (and potentially insertion) events can occur virtually anywhere within the quasi-repetitive units.

[0145] When the quasi-repeated sequences are all ligated in the same orientation, for instance head to tail or vice versa, the cell cannot distinguish individual units. Consequently, the reductive process can occur throughout the sequences. In contrast, when for example, the units are presented head to head, rather than head to tail, the inversion delineates the endpoints of the adjacent unit so that deletion formation will favor the loss of discrete units. Thus, it is preferable with the present method that the sequences are in the same orientation. Random orientation of quasi-repeated sequences will result in the loss of reassortment efficiency, while consistent orientation of the sequences will offer the highest efficiency. However, while having fewer of the contiguous sequences in the same orientation decreases the efficiency, it may still provide sufficient elasticity for the effective recovery of novel



molecules. Constructs can be made with the quasi-repeated sequences in the same orientation to allow higher efficiency.

[0146] Sequences can be assembled in a head to tail orientation using any of a variety of methods, including the following:

[0147] a) Primers that include a poly-A head and poly-T tail which when made single-stranded would provide orientation can be utilized. This is accomplished by having the first few bases of the primers made from RNA and hence easily removed RNaseH.

[0148] b) Primers that include unique restriction cleavage sites can be utilized. Multiple sites, a battery of unique sequences, and repeated synthesis and ligation steps would be required.

[0149] c) The inner few bases of the primer could be thiolated and an exonuclease used to produce properly tailed molecules.

[0150] The recovery of the re-assorted sequences relies on the identification of cloning vectors with a reduced repetitive index (RI). The re-assorted encoding sequences can then be recovered by amplification. The products are re-cloned and expressed. The recovery of cloning vectors with reduced RI can be affected by:

[0151] 1) The use of vectors only stably maintained when the construct is reduced in complexity.

[0152] 2) The physical recovery of shortened vectors by physical procedures. In this case, the cloning vector would be recovered using standard plasmid isolation procedures and size fractionated on either an agarose gel, or column with a low molecular weight cut off utilizing standard procedures.

[0153] 3) The recovery of vectors containing interrupted genes which can be selected when insert size decreases.

[0154] 4) The use of direct selection techniques with an expression vector and the appropriate selection.

[0155] Encoding sequences (for example, genes) from related organisms may demonstrate a high degree of homology and encode quite diverse protein products. These types of sequences are particularly useful in the present invention as quasi-repeats. However, while the examples illustrated below demonstrate the reassortment of nearly identical original encoding sequences (quasi-repeats), this process is not limited to such nearly identical repeats.

[0156] The following example demonstrates a method of the invention. Encoding nucleic acid sequences (quasi-repeats) derived from three (3) unique species are described. Each sequence encodes a protein with a distinct set of properties. Each of the sequences differs by a single or a few base pairs at a unique position in the sequence. The quasi-repeated sequences are separately or collectively amplified and ligated into random assemblies such that all possible permutations and combinations are available in the population of ligated molecules. The number of quasi-repeat units can be controlled by the assembly conditions. The average number of quasi-repeated units in a construct is defined as the repetitive index (RI).

[0157] Once formed, the constructs may, or may not be size fractionated on an agarose gel according to published protocols, inserted into a cloning vector, and transfected into an appropriate host cell. The cells are then propagated and "reductive reassortment" is effected. The rate of the reductive reassortment process may be stimulated by the introduction of DNA damage if desired. Whether the reduction in RI is mediated by deletion formation between repeated sequences by an "intra-molecular" mechanism, or mediated by recombination-like events through "inter-molecular" mechanisms is immaterial. The end result is a reassortment of the molecules into all possible combinations.

[0158] Optionally, the method comprises the additional step of screening the library members of the shuffled pool to identify individual shuffled library members having the ability to bind or otherwise interact, or catalyze a particular reaction (e.g., such as catalytic domain of an enzyme) with a predetermined macromolecule, such as for example a proteinaceous receptor, an oligosaccharide, viron, or other predetermined compound or structure.

[0159] The polypeptides that are identified from such libraries can be used for therapeutic, diagnostic, research and related purposes (e.g., catalysts, solutes for increasing osmolarity of an aqueous solution, and the like), and/or can be subjected to one or more additional cycles of shuffling and/or selection.

[0160] In another aspect, it is envisioned that prior to or during recombination or reassortment, polynucleotides generated by the method of the invention can be subjected to agents or processes which promote the introduction of mutations into the original polynucleotides. The introduction of such mutations would increase the diversity of resulting hybrid polynucleotides and polypeptides encoded therefrom. The agents or processes which promote mutagenesis can include, but are not limited to: (+)-CC-1065, or a synthetic analog such as (+)-CC-1065-(N3-Adenine (See Sun and Hurley, (1992); an N-acetylated or deacetylated 4'-fluoro-4-aminobiphenyl adduct capable of inhibiting DNA synthesis (See, for example, van de Poll et al. (1992)); or a N-acetylated or deacetylated 4-aminobiphenyl adduct capable of inhibiting DNA synthesis (See also, van de Poll et al. (1992), pp. 751-758); trivalent chromium, a trivalent chromium salt, a polycyclic aromatic hydrocarbon (PAH) DNA adduct capable of inhibiting DNA replication, such as 7-bromomethyl-benz[a]anthracene ("BMA"), tris(2,3-dibromopropyl)phosphate ("Tris-BP"), 1,2-dibromo-3-chloropropane ("DBCP"), 2-bromoacrolein (2BA), benzo[a]pyrene-7,8-dihydrodiol-9-10-epoxide ("BPDE"), a platinum(II) halogen salt, N-hydroxy-2-amino-3-methylimidazo[4,5-f]-quinoline ("N-hydroxy-IQ"), and N-hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-f]-pyridine ("N-hydroxy-PhIP"). Especially preferred means for slowing or halting PCR amplification consist of UV light (+)-CC-1065 and (+)-CC-1065-(N3-Adenine). Particularly encompassed means are DNA adducts or polynucleotides comprising the DNA adducts from the polynucleotides or polynucleotides pool, which can be released or removed by a process including heating the solution comprising the polynucleotides prior to further processing.

[0161] In another aspect the invention is directed to a method of producing recombinant proteins having biological activity by treating a sample comprising double-stranded template polynucleotides encoding a wild-type protein under conditions according to the invention which provide for the production of hybrid or re-assorted polynucleotides.

[0162] The invention also provides for the use of proprietary codon primers (containing a degenerate N,N,N sequence) to introduce point mutations into a polynucleotide, so as to generate a set of progeny polypeptides in which a full range of single amino acid substitutions is represented at each amino acid position (gene site saturated mutagenesis (GSSM)). The oligos used are comprised contiguously of a first homologous sequence, a degenerate N,N,N sequence, and preferably but not necessarily a second homologous sequence. The downstream progeny translational products from the use of such oligos include all possible amino acid changes at each amino acid site along the polypeptide, because the degeneracy of the N,N,N sequence includes codons for all 20 amino acids.

[0163] In one aspect, one such degenerate oligo (comprised of one degenerate N,N,N cassette) is used for subjecting each original codon in a parental polynucleotide template to a full range of codon substitutions. In another aspect, at least two degenerate N,N,N cassettes are used – either in the same oligo or not, for subjecting at least two original codons in a parental polynucleotide template to a full range of codon substitutions. Thus, more than one N,N,N sequence can be contained in one oligo to introduce amino acid mutations at more than one site. This plurality of N,N,N sequences can be directly contiguous, or separated by one or more additional nucleotide sequence(s). In another aspect, oligos serviceable for introducing additions and deletions can be used either alone or in combination with the codons containing an N,N,N sequence, to introduce any combination or permutation of amino acid additions, deletions, and/or substitutions.

[0164] In a particular exemplification, it is possible to simultaneously mutagenize two or more contiguous amino acid positions using an oligo that contains contiguous N,N,N triplets, i.e. a degenerate (N,N,N)<sub>n</sub> sequence.

[0165] In another aspect, the present invention provides for the use of degenerate cassettes having less degeneracy than the N,N,N sequence. For example, it may be desirable in some instances to use (e.g. in an oligo) a degenerate triplet sequence comprised of only one N, where said N can be in the first second or third position of the triplet. Any other bases including any combinations and permutations thereof can be used in the remaining two positions of the triplet. Alternatively, it may be desirable in some instances to use (e.g., in an oligo) a degenerate N,N,N triplet sequence, N,N,G/T, or an N,N, G/C triplet sequence.

[0166] It is appreciated, however, that the use of a degenerate triplet (such as N,N,G/T or an N,N, G/C triplet sequence) as disclosed in the instant invention is advantageous for several reasons. In one aspect, this invention provides a means to systematically and fairly easily generate the substitution of the full range of possible amino acids (for a total of 20 amino acids) into each and every amino acid position in a polypeptide. Thus, for a 100 amino acid polypeptide, the invention provides a way to systematically and fairly easily generate 2000 distinct species (i.e., 20 possible amino acids per position times 100 amino acid positions). It is appreciated that there is provided, through the use of an oligo containing a degenerate N,N,G/T or an N,N, G/C triplet sequence, 32 individual sequences that code for 20 possible amino acids. Thus, in a reaction vessel in which a parental polynucleotide sequence is subjected to saturation mutagenesis using one such oligo, there are generated 32 distinct progeny polynucleotides encoding 20 distinct polypeptides. In contrast, the use of a non-degenerate oligo in site-directed mutagenesis leads to only one progeny polypeptide product per reaction vessel.

[0167] This invention also provides for the use of nondegenerate oligos, which can optionally be used in combination with degenerate primers disclosed. It is appreciated that in some situations, it is advantageous to use nondegenerate oligos to generate specific point mutations in a working polynucleotide. This provides a means to generate specific silent point mutations, point mutations leading to corresponding amino acid changes, and point mutations that cause the generation of stop codons and the corresponding expression of polypeptide fragments.

[0168] Thus, in a preferred embodiment of this invention, each saturation mutagenesis reaction vessel contains polynucleotides encoding at least 20 progeny polypeptide molecules such that all 20 amino acids are represented at the one specific amino acid position corresponding to the codon position mutagenized in the parental polynucleotide. The 32-fold degenerate progeny polypeptides generated from each saturation mutagenesis reaction vessel can be subjected to clonal amplification (e.g., cloned into a suitable *E. coli* host using an expression vector) and subjected to expression screening. When an individual progeny polypeptide is identified by screening to display a favorable change in property (when compared to the parental polypeptide), it can be sequenced to identify the correspondingly favorable amino acid substitution contained therein.

[0169] It is appreciated that upon mutagenizing each and every amino acid position in a parental polypeptide using saturation mutagenesis as disclosed herein, favorable amino acid changes may be identified at more than one amino acid position. One or more new progeny molecules can be generated that contain a combination of all or part of these favorable amino acid substitutions. For example, if 2 specific favorable amino acid changes are identified in each of 3 amino acid positions in a polypeptide, the permutations include 3 possibilities at each position (no change from the original amino acid, and each of two favorable changes) and 3 positions. Thus, there are  $3 \times 3 \times 3$  or 27 total possibilities, including 7 that were previously examined - 6 single point mutations (i.e., 2 at each of three positions) and no change at any position.

[0170] In yet another aspect, site-saturation mutagenesis can be used together with shuffling, chimerization, recombination and other mutagenizing processes, along with screening. This invention provides for the use of any mutagenizing process(es), including saturation mutagenesis, in an iterative manner. In one exemplification, the iterative use of any mutagenizing process(es) is used in combination with screening.

[0171] Thus, in a non-limiting exemplification, this invention provides for the use of saturation mutagenesis in combination with additional mutagenization processes, such as process where two or more related polynucleotides are introduced into a suitable host cell such that a hybrid polynucleotide is generated by recombination and reductive reassortment.

[0172] In addition to performing mutagenesis along the entire sequence of a gene, the instant invention provides that mutagenesis can be use to replace each of any number of bases in a polynucleotide sequence, wherein the number of bases to be mutagenized is preferably every integer from 15 to 100,000. Thus, instead of mutagenizing every position along a molecule, one can subject every or a discrete number of bases (preferably a subset totaling from 15 to 100,000) to mutagenesis. Preferably, a separate nucleotide is used for mutagenizing each position or group of positions along a polynucleotide sequence. A group of 3 positions to be mutagenized may be a codon. The mutations are preferably introduced using a mutagenic primer, containing a heterologous cassette, also referred to as a mutagenic cassette. Preferred cassettes can have from 1 to 500 bases. Each nucleotide position in such heterologous cassettes be N, A, C, G, T, A/C, A/G, A/T, C/G, C/T, G/T, C/G/T, A/G/T,

A/C/T, A/C/G, or E, where E is any base that is not A, C, G, or T (E can be referred to as a designer oligo).

[0173] In a general sense, saturation mutagenesis is comprised of mutagenizing a complete set of mutagenic cassettes (wherein each cassette is preferably about 1-500 bases in length) in defined polynucleotide sequence to be mutagenized (wherein the sequence to be mutagenized is preferably from about 15 to 100,000 bases in length). Thus, a group of mutations (ranging from 1 to 100 mutations) is introduced into each cassette to be mutagenized. A grouping of mutations to be introduced into one cassette can be different or the same from a second grouping of mutations to be introduced into a second cassette during the application of one round of saturation mutagenesis. Such groupings are exemplified by deletions, additions, groupings of particular codons, and groupings of particular nucleotide cassettes.

[0174] Defined sequences to be mutagenized include a whole gene, pathway, cDNA, an entire open reading frame (ORF), and entire promoter, enhancer, repressor/transactivator, origin of replication, intron, operator, or any polynucleotide functional group. Generally, a "defined sequences" for this purpose may be any polynucleotide that a 15 base-polynucleotide sequence, and polynucleotide sequences of lengths between 15 bases and 15,000 bases (this invention specifically names every integer in between). Considerations in choosing groupings of codons include types of amino acids encoded by a degenerate mutagenic cassette.

[0175] In a particularly preferred exemplification a grouping of mutations that can be introduced into a mutagenic cassette, this invention specifically provides for degenerate codon substitutions (using degenerate oligos) that code for 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, and 20 amino acids at each position, and a library of polypeptides encoded thereby.

[0176] One aspect of the invention is an isolated nucleic acid comprising one of the sequences of Group A nucleic acid sequences, and sequences substantially identical thereto, the sequences complementary thereto, or a fragment comprising at least 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, 150, 200, 300, 400, or 500 consecutive bases of one of the sequences of a Group A nucleic acid sequence (or the sequences complementary thereto). The isolated,

nucleic acids may comprise DNA, including cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. Alternatively, the isolated nucleic acids may comprise RNA.

[0177] As discussed in more detail below, the isolated nucleic acids of one of the Group A nucleic acid sequences, and sequences substantially identical thereto, may be used to prepare one of the polypeptides of a Group B amino acid sequence, and sequences substantially identical thereto, or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids of one of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto.

[0178] Accordingly, another aspect of the invention is an isolated nucleic acid which encodes one of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids of one of the polypeptides of the Group B amino acid sequences. The coding sequences of these nucleic acids may be identical to one of the coding sequences of one of the nucleic acids of Group A nucleic acid sequences, or a fragment thereof or may be different coding sequences which encode one of the polypeptides of Group B amino acid sequences, sequences substantially identical thereto, and fragments having at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids of one of the polypeptides of Group B amino acid sequences, as a result of the redundancy or degeneracy of the genetic code. The genetic code is well known to those of skill in the art and can be obtained, for example, on page 214 of B. Lewin, Genes VI, Oxford University Press, 1997, the disclosure of which is incorporated herein by reference.

[0179] The isolated nucleic acid which encodes one of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, may include, but is not limited to: only the coding sequence of one of Group A nucleic acid sequences, and sequences substantially identical thereto, and additional coding sequences, such as leader sequences or proprotein sequences and non-coding sequences, such as introns or non-coding sequences 5' and/or 3' of the coding sequence. Thus, as used herein, the term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only



the coding sequence for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequence.

[0180] Alternatively, the nucleic acid sequences of Group A nucleic acid sequences, and sequences substantially identical thereto, may be mutagenized using conventional techniques, such as site directed mutagenesis, or other techniques familiar to those skilled in the art, to introduce silent changes into the polynucleotides of Group A nucleic acid sequences, and sequences substantially identical thereto. As used herein, "silent changes" include, for example, changes which do not alter the amino acid sequence encoded by the polynucleotide. Such changes may be desirable in order to increase the level of the polypeptide produced by host cells containing a vector encoding the polypeptide by introducing codons or codon pairs which occur frequently in the host organism.

[0181] The invention also relates to polynucleotides which have nucleotide changes which result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto. Such nucleotide changes may be introduced using techniques such as site directed mutagenesis, random chemical mutagenesis, exonuclease III deletion, and other recombinant DNA techniques. Alternatively, such nucleotide changes may be naturally occurring allelic variants which are isolated by identifying nucleic acids which specifically hybridize to probes comprising at least 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, 150, 200, 300, 400, or 500 consecutive bases of one of the sequences of Group A nucleic acid sequences, and sequences substantially identical thereto (or the sequences complementary thereto) under conditions of high, moderate, or low stringency as provided herein.

[0182] The isolated nucleic acids of Group A nucleic acid sequences, and sequences substantially identical thereto, the sequences complementary thereto, or a fragment comprising at least 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, 150, 200, 300, 400, or 500 consecutive bases of one of the sequences of Group A nucleic acid sequences, and sequences substantially identical thereto, or the sequences complementary thereto may also be used as probes to determine whether a biological sample, such as a soil sample, contains an organism having a nucleic acid sequence of the invention or an organism from which the nucleic acid was obtained. In such procedures, a biological sample potentially harboring the organism

from which the nucleic acid was isolated is obtained and nucleic acids are obtained from the sample. The nucleic acids are contacted with the probe under conditions which permit the probe to specifically hybridize to any complementary sequences from which are present therein.

[0183] Where necessary, conditions which permit the probe to specifically hybridize to complementary sequences may be determined by placing the probe in contact with complementary sequences from samples known to contain the complementary sequence as well as control sequences which do not contain the complementary sequence. Hybridization conditions, such as the salt concentration of the hybridization buffer, the formamide concentration of the hybridization buffer, or the hybridization temperature, may be varied to identify conditions which allow the probe to hybridize specifically to complementary nucleic acids.

[0184] If the sample contains the organism from which the nucleic acid was isolated, specific hybridization of the probe is then detected. Hybridization may be detected by labeling the probe with a detectable agent such as a radioactive isotope, a fluorescent dye or an enzyme capable of catalyzing the formation of a detectable product.

[0185] Many methods for using the labeled probes to detect the presence of complementary nucleic acids in a sample are familiar to those skilled in the art. These include Southern Blots, Northern Blots, colony hybridization procedures, and dot blots. Protocols for each of these procedures are provided in Ausubel et al. Current Protocols in Molecular Biology, John Wiley 503 Sons, Inc. (1997) and Sambrook et al., Molecular Cloning: A Laboratory Manual 2nd Ed., Cold Spring Harbor Laboratory Press (1989), the entire disclosures of which are incorporated herein by reference.

[0186] Alternatively, more than one probe (at least one of which is capable of specifically hybridizing to any complementary sequences which are present in the nucleic acid sample), may be used in an amplification reaction to determine whether the sample contains an organism containing a nucleic acid sequence of the invention (e.g., an organism from which the nucleic acid was isolated). Typically, the probes comprise oligonucleotides. In one embodiment, the amplification reaction may comprise a PCR reaction. PCR protocols are described in Ausubel and Sambrook, supra. Alternatively, the amplification may

comprise a ligase chain reaction, 3SR, or strand displacement reaction. (See Barany, F., "The Ligase Chain Reaction in a PCR World", PCR Methods and Applications 1:5-16, 1991; E. Fahy et al., "Self-sustained Sequence Replication (3SR): An Isothermal Transcription-based Amplification System Alternative to PCR", PCR Methods and Applications 1:25-33, 1991; and Walker G.T. et al., "Strand Displacement Amplification-an Isothermal *in vitro* DNA Amplification Technique", Nucleic Acid Research 20:1691-1696, 1992, the disclosures of which are incorporated herein by reference in their entireties). In such procedures, the nucleic acids in the sample are contacted with the probes, the amplification reaction is performed, and any resulting amplification product is detected. The amplification product may be detected by performing gel electrophoresis on the reaction products and staining the gel with an intercalator such as ethidium bromide. Alternatively, one or more of the probes may be labeled with a radioactive isotope and the presence of a radioactive amplification product may be detected by autoradiography after gel electrophoresis.

[0187] Probes derived from sequences near the ends of the sequences of Group A nucleic acid sequences, and sequences substantially identical thereto, may also be used in chromosome walking procedures to identify clones containing genomic sequences located adjacent to the sequences of Group A nucleic acid sequences, and sequences substantially identical thereto. Such methods allow the isolation of genes which encode additional proteins from the host organism.

[0188] The isolated nucleic acids of Group A nucleic acid sequences, and sequences substantially identical thereto, the sequences complementary thereto, or a fragment comprising at least 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, 150, 200, 300, 400, or 500 consecutive bases of one of the sequences of Group A nucleic acid sequences, and sequences substantially identical thereto, or the sequences complementary thereto may be used as probes to identify and isolate related nucleic acids. In some embodiments, the related nucleic acids may be cDNAs or genomic DNAs from organisms other than the one from which the nucleic acid was isolated. For example, the other organisms may be related organisms. In such procedures, a nucleic acid sample is contacted with the probe under conditions which permit the probe to specifically hybridize to related sequences. Hybridization of the probe to nucleic acids from the related organism is then detected using any of the methods described above.

[0189] In nucleic acid hybridization reactions, the conditions used to achieve a particular level of stringency will vary, depending on the nature of the nucleic acids being hybridized. For example, the length, degree of complementarity, nucleotide sequence composition (e.g., GC v. AT content), and nucleic acid type (e.g., RNA v. DNA) of the hybridizing regions of the nucleic acids can be considered in selecting hybridization conditions. An additional consideration is whether one of the nucleic acids is immobilized, for example, on a filter.

[0190] Hybridization may be carried out under conditions of low stringency, moderate stringency or high stringency. As an example of nucleic acid hybridization, a polymer membrane containing immobilized denatured nucleic acids is first prehybridized for 30 minutes at 45°C in a solution consisting of 0.9 M NaCl, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0, 5.0 mM Na<sub>2</sub>EDTA, 0.5% SDS, 10X Denhardt's, and 0.5 mg/ml polyriboadenylic acid. Approximately 2 X 10<sup>7</sup> cpm (specific activity 4-9 X 10<sup>8</sup> cpm/ug) of <sup>32</sup>P end-labeled oligonucleotide probe are then added to the solution. After 12-16 hours of incubation, the membrane is washed for 30 minutes at room temperature in 1X SET (150 mM NaCl, 20 mM Tris hydrochloride, pH 7.8, 1 mM Na<sub>2</sub>EDTA) containing 0.5% SDS, followed by a 30 minute wash in fresh 1X SET at T<sub>m</sub>-10°C for the oligonucleotide probe. The membrane is then exposed to auto-radiographic film for detection of hybridization signals.

[0191] By varying the stringency of the hybridization conditions used to identify nucleic acids, such as cDNAs or genomic DNAs, which hybridize to the detectable probe, nucleic acids having different levels of homology to the probe can be identified and isolated. Stringency may be varied by conducting the hybridization at varying temperatures below the melting temperatures of the probes. The melting temperature, T<sub>m</sub>, is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly complementary probe. Very stringent conditions are selected to be equal to or about 5°C lower than the T<sub>m</sub> for a particular probe. The melting temperature of the probe may be calculated using the following formulas:

[0192] For probes between 14 and 70 nucleotides in length the melting temperature (T<sub>m</sub>) is calculated using the formula:  $T_m = 81.5 + 16.6(\log [Na^+]) + 0.41(\text{fraction G+C}) - (600/N)$  where N is the length of the probe.

[0193] If the hybridization is carried out in a solution containing formamide, the melting temperature may be calculated using the equation:  $T_m = 81.5 + 16.6(\log [Na^+] + 0.41(\text{fraction G+C}) - (0.63\% \text{ formamide}) - (600/N))$  where N is the length of the probe.

[0194] Prehybridization may be carried out in 6X SSC, 5X Denhardt's reagent, 0.5% SDS, 100µg denatured fragmented salmon sperm DNA or 6X SSC, 5X Denhardt's reagent, 0.5% SDS, 100µg denatured fragmented salmon sperm DNA, 50% formamide. The formulas for SSC and Denhardt's solutions are listed in Sambrook et al., supra.

[0195] Hybridization is conducted by adding the detectable probe to the prehybridization solutions listed above. Where the probe comprises double stranded DNA, it is denatured before addition to the hybridization solution. The filter is contacted with the hybridization solution for a sufficient period of time to allow the probe to hybridize to cDNAs or genomic DNAs containing sequences complementary thereto or homologous thereto. For probes over 200 nucleotides in length, the hybridization may be carried out at 15-25°C below the  $T_m$ . For shorter probes, such as oligonucleotide probes, the hybridization may be conducted at 5-10°C below the  $T_m$ . Typically, for hybridizations in 6X SSC, the hybridization is conducted at approximately 68°C. Usually, for hybridizations in 50% formamide containing solutions, the hybridization is conducted at approximately 42°C.

[0196] All of the foregoing hybridizations would be considered to be under conditions of high stringency.

[0197] Following hybridization, the filter is washed to remove any non-specifically bound detectable probe. The stringency used to wash the filters can also be varied depending on the nature of the nucleic acids being hybridized, the length of the nucleic acids being hybridized, the degree of complementarity, the nucleotide sequence composition (e.g., GC v. AT content), and the nucleic acid type (e.g., RNA v. DNA). Examples of progressively higher stringency condition washes are as follows: 2X SSC, 0.1% SDS at room temperature for 15 minutes (low stringency); 0.1X SSC, 0.5% SDS at room temperature for 30 minutes to 1 hour (moderate stringency); 0.1X SSC, 0.5% SDS for 15 to 30 minutes at between the hybridization temperature and 68°C (high stringency); and 0.15M NaCl for 15 minutes at 72°C (very high stringency). A final low stringency wash can be conducted in 0.1X SSC at room temperature. The examples above are merely illustrative of one set of conditions that

can be used to wash filters. One of skill in the art would know that there are numerous recipes for different stringency washes. Some other examples are given below.

[0198] Nucleic acids which have hybridized to the probe are identified by autoradiography or other conventional techniques.

[0199] The above procedure may be modified to identify nucleic acids having decreasing levels of homology to the probe sequence. For example, to obtain nucleic acids of decreasing homology to the detectable probe, less stringent conditions may be used. For example, the hybridization temperature may be decreased in increments of 5°C from 68°C to 42°C in a hybridization buffer having a Na<sup>+</sup> concentration of approximately 1M. Following hybridization, the filter may be washed with 2X SSC, 0.5% SDS at the temperature of hybridization. These conditions are considered to be "moderate" conditions above 50°C and "low" conditions below 50°C. A specific example of "moderate" hybridization conditions is when the above hybridization is conducted at 55°C. A specific example of "low stringency" hybridization conditions is when the above hybridization is conducted at 45°C.

[0200] Alternatively, the hybridization may be carried out in buffers, such as 6X SSC, containing formamide at a temperature of 42°C. In this case, the concentration of formamide in the hybridization buffer may be reduced in 5% increments from 50% to 0% to identify clones having decreasing levels of homology to the probe. Following hybridization, the filter may be washed with 6X SSC, 0.5% SDS at 50°C. These conditions are considered to be "moderate" conditions above 25% formamide and "low" conditions below 25% formamide. A specific example of "moderate" hybridization conditions is when the above hybridization is conducted at 30% formamide. A specific example of "low stringency" hybridization conditions is when the above hybridization is conducted at 10% formamide.

[0201] For example, the preceding methods may be used to isolate nucleic acids having a sequence with at least about 97%, at least 95%, at least 90%, at least 85%, at least 80%, at least 70%, at least 65%, at least 60%, at least 55%, or at least 50% homology to a nucleic acid sequence selected from the group consisting of one of the sequences of Group A nucleic acid sequences, and sequences substantially identical thereto, or fragments comprising at least about 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, 150, 200, 300, 400, or 500 consecutive bases thereof, and the sequences complementary thereto. Homology may be

measured using the alignment algorithm. For example, the homologous polynucleotides may have a coding sequence which is a naturally occurring allelic variant of one of the coding sequences described herein. Such allelic variants may have a substitution, deletion or addition of one or more nucleotides when compared to the nucleic acids of Group A nucleic acid sequences or the sequences complementary thereto.

[0202] Additionally, the above procedures may be used to isolate nucleic acids which encode polypeptides having at least about 99%, 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 65%, at least 60%, at least 55%, or at least 50% homology to a polypeptide having the sequence of one of Group B amino acid sequences, and sequences substantially identical thereto, or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof as determined using a sequence alignment algorithm (e.g., such as the FASTA version 3.0t78 algorithm with the default parameters).

[0203] Another aspect of the invention is an isolated or purified polypeptide comprising the sequence of one of Group A nucleic acid sequences, and sequences substantially identical thereto, or fragments comprising at least about 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof. As discussed above, such polypeptides may be obtained by inserting a nucleic acid encoding the polypeptide into a vector such that the coding sequence is operably linked to a sequence capable of driving the expression of the encoded polypeptide in a suitable host cell. For example, the expression vector may comprise a promoter, a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

[0100] Promoters suitable for expressing the polypeptide or fragment thereof in bacteria include the *E. coli lac* or *trp* promoters, the *lacI* promoter, the *lacZ* promoter, the *T3* promoter, the *T7* promoter, the *gpt* promoter, the *lambda P<sub>R</sub>* promoter, the *lambda P<sub>L</sub>* promoter, promoters from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), and the acid phosphatase promoter. Fungal promoters include the  $\nabla$  factor promoter. Eukaryotic promoters include the CMV immediate early promoter, the HSV thymidine kinase promoter, heat shock promoters, the early and late SV40 promoter, LTRs

from retroviruses, and the mouse metallothionein-I promoter. Other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses may also be used.

[0204] Mammalian expression vectors may also comprise an origin of replication, any necessary ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. In some embodiments, DNA sequences derived from the SV40 splice and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

[0205] Vectors for expressing the polypeptide or fragment thereof in eukaryotic cells may also contain enhancers to increase expression levels. Enhancers are cis-acting elements of DNA, usually from about 10 to about 300 bp in length that act on a promoter to increase its transcription. Examples include the SV40 enhancer on the late side of the replication origin bp 100 to 270, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and the adenovirus enhancers.

[0206] In addition, the expression vectors typically contain one or more selectable marker genes to permit selection of host cells containing the vector. Such selectable markers include genes encoding dihydrofolate reductase or genes conferring neomycin resistance for eukaryotic cell culture, genes conferring tetracycline or ampicillin resistance in *E. coli*, and the *S. cerevisiae* TRP1 gene.

[0207] In some embodiments, the nucleic acid encoding one of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or fragments comprising at least about 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof is assembled in appropriate phase with a leader sequence capable of directing secretion of the translated polypeptide or fragment thereof. Optionally, the nucleic acid can encode a fusion polypeptide in which one of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof is fused to heterologous peptides or polypeptides, such as N-terminal identification peptides which impart desired characteristics, such as increased stability or simplified purification.



[0208] The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is ligated to the desired position in the vector following digestion of the insert and the vector with appropriate restriction endonucleases. Alternatively, blunt ends in both the insert and the vector may be ligated. A variety of cloning techniques are disclosed in Ausubel et al. Current Protocols in Molecular Biology, John Wiley 503 Sons, Inc. 1997 and Sambrook et al., Molecular Cloning: A Laboratory Manual 2nd Ed., Cold Spring Harbor Laboratory Press (1989), the entire disclosures of which are incorporated herein by reference. Such procedures and others are deemed to be within the scope of those skilled in the art.

[0209] The vector may be, for example, in the form of a plasmid, a viral particle, or a phage. Other vectors include chromosomal, nonchromosomal and synthetic DNA sequences, derivatives of SV40; bacterial plasmids, phage DNA, baculovirus, yeast plasmids, vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. A variety of cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.

[0210] Particular bacterial vectors which may be used include the commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017), pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden), GEM1 (Promega Biotec, Madison, WI, USA) pQE70, pQE60, pQE-9 (Qiagen), pD10, psiX174 pBluescript II KS, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene), ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia), pKK232-8 and pCM7. Particular eukaryotic vectors include pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, and pSVL (Pharmacia). However, any other vector may be used as long as it is replicable and viable in the host cell.

[0211] The host cell may be any of the host cells familiar to those skilled in the art, including prokaryotic cells, eukaryotic cells, mammalian cells, insect cells, or plant cells. As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as *E. coli*, *Streptomyces*, *Bacillus subtilis*, *Salmonella typhimurium* and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, fungal cells, such as yeast,

insect cells such as *Drosophila* S2 and *Spodoptera* Sf9, animal cells such as CHO, COS or Bowes melanoma, and adenoviruses. The selection of an appropriate host is within the abilities of those skilled in the art.

[0212] The vector may be introduced into the host cells using any of a variety of techniques, including transformation, transfection, transduction, viral infection, gene guns, or Ti-mediated gene transfer. Particular methods include calcium phosphate transfection, DEAE-Dextran mediated transfection, lipofection, or electroporation (Davis, L., Dibner, M., Battey, I., *Basic Methods in Molecular Biology*, (1986)).

[0213] Where appropriate, the engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the genes of the invention. Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter may be induced by appropriate means (e.g., temperature shift or chemical induction) and the cells may be cultured for an additional period to allow them to produce the desired polypeptide or fragment thereof.

[0214] Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract is retained for further purification. Microbial cells employed for expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents. Such methods are well known to those skilled in the art. The expressed polypeptide or fragment thereof can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the polypeptide. If desired, high performance liquid chromatography (HPLC) can be employed for final purification steps.

[0215] Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts (described by Gluzman, *Cell*, 23:175, 1981), and other cell

lines capable of expressing proteins from a compatible vector, such as the C127, 3T3, CHO, HeLa and BHK cell lines.

[0216] The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Depending upon the host employed in a recombinant production procedure, the polypeptides produced by host cells containing the vector may be glycosylated or may be non-glycosylated. Polypeptides of the invention may or may not also include an initial methionine amino acid residue.

[0217] Alternatively, the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof can be synthetically produced by conventional peptide synthesizers. In other embodiments, fragments or portions of the polypeptides may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length polypeptides.

[0218] Cell-free translation systems can also be employed to produce one of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof using mRNAs transcribed from a DNA construct comprising a promoter operably linked to a nucleic acid encoding the polypeptide or fragment thereof. In some embodiments, the DNA construct may be linearized prior to conducting an *in vitro* transcription reaction. The transcribed mRNA is then incubated with an appropriate cell-free translation extract, such as a rabbit reticulocyte extract, to produce the desired polypeptide or fragment thereof.

[0219] The invention also relates to variants of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof. The term "variant" includes derivatives or analogs of these polypeptides. In particular, the variants may differ in amino acid sequence from the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, by one or more substitutions, additions, deletions, fusions and truncations, which may be present in any combination.

[0220] The variants may be naturally occurring or created *in vitro*. In particular, such variants may be created using genetic engineering techniques such as site directed mutagenesis, random chemical mutagenesis, Exonuclease III deletion procedures, and standard cloning techniques. Alternatively, such variants, fragments, analogs, or derivatives may be created using chemical synthesis or modification procedures.

[0221] Other methods of making variants are also familiar to those skilled in the art. These include procedures in which nucleic acid sequences obtained from natural isolates are modified to generate nucleic acids which encode polypeptides having characteristics which enhance their value in industrial or laboratory applications. In such procedures, a large number of variant sequences having one or more nucleotide differences with respect to the sequence obtained from the natural isolate are generated and characterized. Typically, these nucleotide differences result in amino acid changes with respect to the polypeptides encoded by the nucleic acids from the natural isolates.

[0222] For example, variants may be created using error prone PCR. In error prone PCR, PCR is performed under conditions where the copying fidelity of the DNA polymerase is low, such that a high rate of point mutations is obtained along the entire length of the PCR product. Error prone PCR is described in Leung, D.W., et al., Technique, 1:11-15, 1989) and Caldwell, R. C. & Joyce G.F., PCR Methods Applic., 2:28-33, 1992, the disclosure of which is incorporated herein by reference in its entirety. Briefly, in such procedures, nucleic acids to be mutagenized are mixed with PCR primers, reaction buffer, MgCl<sub>2</sub>, MnCl<sub>2</sub>, Taq polymerase and an appropriate concentration of dNTPs for achieving a high rate of point mutation along the entire length of the PCR product. For example, the reaction may be performed using 20 fmoles of nucleic acid to be mutagenized, 30pmole of each PCR primer, a reaction buffer comprising 50mM KCl, 10mM Tris HCl (pH 8.3) and 0.01% gelatin, 7mM MgCl<sub>2</sub>, 0.5mM MnCl<sub>2</sub>, 5 units of Taq polymerase, 0.2mM dGTP, 0.2mM dATP, 1mM dCTP, and 1mM dTTP. PCR may be performed for 30 cycles of 94° C for 1 min, 45° C for 1 min, and 72° C for 1 min. However, it will be appreciated that these parameters may be varied as appropriate. The mutagenized nucleic acids are cloned into an appropriate vector and the activities of the polypeptides encoded by the mutagenized nucleic acids is evaluated.

[0223] Variants may also be created using oligonucleotide directed mutagenesis to generate site-specific mutations in any cloned DNA of interest. Oligonucleotide mutagenesis is described in Reidhaar-Olson, J.F. & Sauer, R.T., et al., Science, 241:53-57, 1988, the disclosure of which is incorporated herein by reference in its entirety. Briefly, in such procedures a plurality of double stranded oligonucleotides bearing one or more mutations to be introduced into the cloned DNA are synthesized and inserted into the cloned DNA to be mutagenized. Clones containing the mutagenized DNA are recovered and the activities of the polypeptides they encode are assessed.

[0224] Another method for generating variants is assembly PCR. Assembly PCR involves the assembly of a PCR product from a mixture of small DNA fragments. A large number of different PCR reactions occur in parallel in the same vial, with the products of one reaction priming the products of another reaction. Assembly PCR is described in U.S. Patent No. 5,965,408, filed July 9, 1996, entitled, "Method of DNA Reassembly by Interrupting Synthesis", the disclosure of which is incorporated herein by reference in its entirety.

[0225] Still another method of generating variants is sexual PCR mutagenesis. In sexual PCR mutagenesis, forced homologous recombination occurs between DNA molecules of different but highly related DNA sequence *in vitro*, as a result of random fragmentation of the DNA molecule based on sequence homology, followed by fixation of the crossover by primer extension in a PCR reaction. Sexual PCR mutagenesis is described in Stemmer, W.P., PNAS, USA, 91:10747-10751, 1994, the disclosure of which is incorporated herein by reference. Briefly, in such procedures a plurality of nucleic acids to be recombined are digested with DNase to generate fragments having an average size of 50-200 nucleotides. Fragments of the desired average size are purified and resuspended in a PCR mixture. PCR is conducted under conditions which facilitate recombination between the nucleic acid fragments. For example, PCR may be performed by resuspending the purified fragments at a concentration of 10-30ng/:l in a solution of 0.2mM of each dNTP, 2.2mM MgCl<sub>2</sub>, 50mM KCL, 10mM Tris HCl, pH 9.0, and 0.1% Triton X-100. 2.5 units of Taq polymerase per 100:1 of reaction mixture is added and PCR is performed using the following regime: 94° C for 60 seconds, 94° C for 30 seconds, 50-55° C for 30 seconds, 72° C for 30 seconds (30-45 times) and 72° C for 5 minutes. However, it will be appreciated that these parameters may be varied as appropriate. In some embodiments, oligonucleotides may be included in the PCR

reactions. In other embodiments, the Klenow fragment of DNA polymerase I may be used in a first set of PCR reactions and Taq polymerase may be used in a subsequent set of PCR reactions. Recombinant sequences are isolated and the activities of the polypeptides they encode are assessed.

[0226] Variants may also be created by *in vivo* mutagenesis. In some embodiments, random mutations in a sequence of interest are generated by propagating the sequence of interest in a bacterial strain, such as an *E. coli* strain, which carries mutations in one or more of the DNA repair pathways. Such "mutator" strains have a higher random mutation rate than that of a wild-type parent. Propagating the DNA in one of these strains will eventually generate random mutations within the DNA. Mutator strains suitable for use for *in vivo* mutagenesis are described in PCT Publication No. WO 91/16427, published October 31, 1991, entitled "Methods for Phenotype Creation from Multiple Gene Populations" the disclosure of which is incorporated herein by reference in its entirety.

[0227] Variants may also be generated using cassette mutagenesis. In cassette mutagenesis a small region of a double stranded DNA molecule is replaced with a synthetic oligonucleotide "cassette" that differs from the native sequence. The oligonucleotide often contains completely and/or partially randomized native sequence.

[0228] Recursive ensemble mutagenesis may also be used to generate variants. Recursive ensemble mutagenesis is an algorithm for protein engineering (protein mutagenesis) developed to produce diverse populations of phenotypically related mutants whose members differ in amino acid sequence. This method uses a feedback mechanism to control successive rounds of combinatorial cassette mutagenesis. Recursive ensemble mutagenesis is described in Arkin, A.P. and Youvan, D.C., PNAS, USA, 89:7811-7815, 1992, the disclosure of which is incorporated herein by reference in its entirety.

[0229] In some embodiments, variants are created using exponential ensemble mutagenesis. Exponential ensemble mutagenesis is a process for generating combinatorial libraries with a high percentage of unique and functional mutants, wherein small groups of residues are randomized in parallel to identify, at each altered position, amino acids which lead to functional proteins. Exponential ensemble mutagenesis is described in Delegrave, S. and Youvan, D.C., Biotechnology Research, 11:1548-1552, 1993, the disclosure of which

incorporated herein by reference in its entirety. Random and site-directed mutagenesis are described in Arnold, F.H., Current Opinion in Biotechnology, 4:450-455, 1993, the disclosure of which is incorporated herein by reference in its entirety.

[0230] In some embodiments, the variants are created using shuffling procedures wherein portions of a plurality of nucleic acids which encode distinct polypeptides are fused together to create chimeric nucleic acid sequences which encode chimeric polypeptides as described in U.S. Patent No. 5,965,408, filed July 9, 1996, entitled, "Method of DNA Reassembly by Interrupting Synthesis", and U.S. Patent No. 5,939,250, filed May 22, 1996, entitled, "Production of Enzymes Having Desired Activities by Mutagenesis", both of which are incorporated herein by reference.

[0231] The variants of the polypeptides of Group B amino acid sequences may be variants in which one or more of the amino acid residues of the polypeptides of the Group B amino acid sequences are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code.

[0232] Conservative substitutions are those that substitute a given amino acid in a polypeptide by another amino acid of like characteristics. Typically seen as conservative substitutions are the following replacements: replacements of an aliphatic amino acid such as Alanine, Valine, Leucine and Isoleucine with another aliphatic amino acid; replacement of a Serine with a Threonine or vice versa; replacement of an acidic residue such as Aspartic acid and Glutamic acid with another acidic residue; replacement of a residue bearing an amide group, such as Asparagine and Glutamine, with another residue bearing an amide group; exchange of a basic residue such as Lysine and Arginine with another basic residue; and replacement of an aromatic residue such as Phenylalanine, Tyrosine with another aromatic residue.

[0233] Other variants are those in which one or more of the amino acid residues of the polypeptides of the Group B amino acid sequences includes a substituent group.

[0234] Still other variants are those in which the polypeptide is associated with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol).

[0235] Additional variants are those in which additional amino acids are fused to the polypeptide, such as a leader sequence, a secretory sequence, a proprotein sequence or a sequence which facilitates purification, enrichment, or stabilization of the polypeptide.

[0236] In some embodiments, the fragments, derivatives and analogs retain the same biological function or activity as the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto. In other embodiments, the fragment, derivative, or analog includes a proprotein, such that the fragment, derivative, or analog can be activated by cleavage of the proprotein portion to produce an active polypeptide.

[0237] Another aspect of the invention is polypeptides or fragments thereof which have at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more than about 95% homology to one of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or a fragment comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof. Homology may be determined using any of the programs described above which aligns the polypeptides or fragments being compared and determines the extent of amino acid identity or similarity between them. It will be appreciated that amino acid "homology" includes conservative amino acid substitutions such as those described above.

[0238] The polypeptides or fragments having homology to one of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or a fragment comprising at least about 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof may be obtained by isolating the nucleic acids encoding them using the techniques described above.

[0239] Alternatively, the homologous polypeptides or fragments may be obtained through biochemical enrichment or purification procedures. The sequence of potentially homologous polypeptides or fragments may be determined by proteolytic digestion, gel



electrophoresis and/or microsequencing. The sequence of the prospective homologous polypeptide or fragment can be compared to one of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or a fragment comprising at least about 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof using any of the programs described above.

[0240] Another aspect of the invention is an assay for identifying fragments or variants of Group B amino acid sequences, and sequences substantially identical thereto, which retain the enzymatic function of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto. For example the fragments or variants of said polypeptides, may be used to catalyze biochemical reactions, which indicate that the fragment or variant retains the enzymatic activity of the polypeptides in the Group B amino acid sequences.

[0241] The assay for determining if fragments of variants retain the enzymatic activity of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto includes the steps of: contacting the polypeptide fragment or variant with a substrate molecule under conditions which allow the polypeptide fragment or variant to function, and detecting either a decrease in the level of substrate or an increase in the level of the specific reaction product of the reaction between the polypeptide and substrate.

[0242] The polypeptides of Group B amino acid sequences, and sequences substantially identical thereto or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof may be used in a variety of applications. For example, the polypeptides or fragments thereof may be used to catalyze biochemical reactions. In accordance with one aspect of the invention, there is provided a process for utilizing the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto or polynucleotides encoding such polypeptides for hydrolyzing glycosidic linkages. In such procedures, a substance containing a glycosidic linkage (e.g., a starch) is contacted with one of the polypeptides of Group B amino acid sequences, or sequences substantially identical thereto under conditions which facilitate the hydrolysis of the glycosidic linkage.

[0243] The polypeptides of Group B amino acid sequences, and sequences substantially identical thereto or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof, may also be used in the liquefaction and saccharification of starch. Using the polypeptides or fragments thereof of this invention, liquefaction may be carried out at a lower pH than with previous enzymes. In one embodiment, liquefaction is performed at a pH of 4.5. Additionally, the polypeptides or fragments thereof of this invention are less calcium dependent than enzymes previously used in these processes. In liquefaction amylases are used to hydrolyze starch. In a preferred embodiment, the polypeptides or fragments thereof of this invention are thermostable at 90-95°C.

[0244] The polypeptides of Group B amino acid sequences, and sequences substantially identical thereto or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof, may also be used to generate antibodies which bind specifically to the polypeptides or fragments. The resulting antibodies may be used in immunoaffinity chromatography procedures to isolate or purify the polypeptide or to determine whether the polypeptide is present in a biological sample. In such procedures, a protein preparation, such as an extract, or a biological sample is contacted with an antibody capable of specifically binding to one of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof.

[0245] In immunoaffinity procedures, the antibody is attached to a solid support, such as a bead or other column matrix. The protein preparation is placed in contact with the antibody under conditions in which the antibody specifically binds to one of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or fragment thereof. After a wash to remove non-specifically bound proteins, the specifically bound polypeptides are eluted.

[0246] The ability of proteins in a biological sample to bind to the antibody may be determined using any of a variety of procedures familiar to those skilled in the art. For example, binding may be determined by labeling the antibody with a detectable label such as a fluorescent agent, an enzymatic label, or a radioisotope. Alternatively, binding of the

antibody to the sample may be detected using a secondary antibody having such a detectable label thereon. Particular assays include ELISA assays, sandwich assays, radioimmunoassays, and Western Blots.

[0247] Polyclonal antibodies generated against the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof can be obtained by direct injection of the polypeptides into an animal or by administering the polypeptides to an animal, for example, a nonhuman. The antibody so obtained will then bind the polypeptide itself. In this manner, even a sequence encoding only a fragment of the polypeptide can be used to generate antibodies which may bind to the whole native polypeptide. Such antibodies can then be used to isolate the polypeptide from cells expressing that polypeptide.

[0248] For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, Nature, 256:495-497, 1975, the disclosure of which is incorporated herein by reference), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., Immunology Today 4:72, 1983, the disclosure of which is incorporated herein by reference), and the EBV-hybridoma technique (Cole, et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96, the disclosure of which is incorporated herein by reference).

[0249] Techniques described for the production of single chain antibodies (U.S. Patent No. 4,946,778, the disclosure of which is incorporated herein by reference) can be adapted to produce single chain antibodies to the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof. Alternatively, transgenic mice may be used to express humanized antibodies to these polypeptides or fragments thereof.

[0250] Antibodies generated against the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof may be used in

screening for similar polypeptides from other organisms and samples. In such techniques, polypeptides from the organism are contacted with the antibody and those polypeptides which specifically bind the antibody are detected. Any of the procedures described above may be used to detect antibody binding. One such screening assay is described in "Methods for Measuring Cellulase Activities", Methods in Enzymology, Vol 160, pp. 87-116, which is hereby incorporated by reference in its entirety.

[0251] As used herein the term "nucleic acid sequence as set forth in SEQ ID Nos.: 1, 3, 5, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 251, 253, 255, 257, 259, 261, 263, 265, 267, 269, 271, 273, 275, 277, 279, 281, 283, 285, 287, 289, 291, 293, 295, 297, 299" encompasses the nucleotide sequences of Group A nucleic acid sequences, and sequences substantially identical thereto, as well as sequences homologous to Group A nucleic acid sequences, and fragments thereof and sequences complementary to all of the preceding sequences. The fragments include portions of SEQ ID Nos.: 1, 3, 5, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 251, 253, 255, 257, 259, 261, 263, 265, 267, 269, 271, 273, 275, 277, 279, 281, 283, 285, 287, 289, 291, 293, 295, 297, and 299, comprising at least 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, 150, 200, 300, 400, or 500 consecutive nucleotides of Group A nucleic acid sequences, and sequences substantially identical thereto. Homologous sequences and fragments of Group A nucleic acid sequences, and sequences substantially identical thereto, refer to a sequence having at least 99%, 98%, 97%, 96%, 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55% or 50% homology to these sequences. Homology may be determined using

any of the computer programs and parameters described herein, including FASTA version 3.0t78 with the default parameters. Homologous sequences also include RNA sequences in which uridines replace the thymines in the nucleic acid sequences as set forth in the Group A nucleic acid sequences. The homologous sequences may be obtained using any of the procedures described herein or may result from the correction of a sequencing error. It will be appreciated that the nucleic acid sequences as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, can be represented in the traditional single character format (See the inside back cover of Stryer, Lubert. Biochemistry, 3rd Ed., W. H. Freeman & Co., New York.) or in any other format which records the identity of the nucleotides in a sequence.

[0252] As used herein the term "a polypeptide sequence as set forth in SEQ ID Nos: 2, 4, 6, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 252, 254, 256, 258, 260, 262, 264, 266, 268, 270, 272, 274, 276, 278, 280, 282, 284, 286, 288, 290, 292, 294, 296, 298" encompasses the polypeptide sequence of Group B amino acid sequences, and sequences substantially identical thereto, which are encoded by a sequence as set forth in SEQ ID Nos: 2, 4, 6, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 252, 254, 256, 258, 260, 262, 264, 266, 268, 270, 272, 274, 276, 278, 280, 282, 284, 286, 288, 290, 292, 294, 296, 298, polypeptide sequences homologous to the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or fragments of any of the preceding sequences. Homologous polypeptide sequences refer to a polypeptide sequence having at least 99%, 98%, 97%, 96%,

95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55% or 50% homology to one of the polypeptide sequences of the Group B amino acid sequences. Homology may be determined using any of the computer programs and parameters described herein, including FASTA version 3.0t78 with the default parameters or with any modified parameters. The homologous sequences may be obtained using any of the procedures described herein or may result from the correction of a sequencing error. The polypeptide fragments comprise at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto. It will be appreciated that the polypeptide codes as set forth in Group B amino acid sequences, and sequences substantially identical thereto, can be represented in the traditional single character format or three letter format (See the inside back cover of Stryer, Lubert. Biochemistry, 3rd Ed., W. H Freeman & Co., New York.) or in any other format which relates the identity of the polypeptides in a sequence.

[0253] It will be appreciated by those skilled in the art that a nucleic acid sequence as set forth in SEQ ID No.s: 1, 3, 5, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 251, 253, 255, 257, 259, 261, 263, 265, 267, 269, 271, 273, 275, 277, 279, 281, 283, 285, 287, 289, 291, 293, 295, 297, 299 and a polypeptide sequence as set forth in SEQ ID No.s: 2, 4, 6, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 252, 254, 256, 258, 260, 262, 264, 266, 268, 270, 272, 274, 276, 278, 280, 282, 284, 286, 288, 290, 292, 294, 296, 298 can be stored, recorded, and manipulated on any medium which can be read and accessed by a computer. As used herein,

the words "recorded" and "stored" refer to a process for storing information on a computer medium. A skilled artisan can readily adopt any of the presently known methods for recording information on a computer readable medium to generate manufactures comprising one or more of the nucleic acid sequences as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, one or more of the polypeptide sequences as set forth in Group B amino acid sequences, and sequences substantially identical thereto. Another aspect of the invention is a computer readable medium having recorded thereon at least 2, 5, 10, 15, or 20 nucleic acid sequences as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto.

[0254] Another aspect of the invention is a computer readable medium having recorded thereon one or more of the nucleic acid sequences as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto. Another aspect of the invention is a computer readable medium having recorded thereon one or more of the polypeptide sequences as set forth in Group B amino acid sequences, and sequences substantially identical thereto. Another aspect of the invention is a computer readable medium having recorded thereon at least 2, 5, 10, 15, or 20 of the sequences as set forth above.

[0255] Computer readable media include magnetically readable media, optically readable media, electronically readable media and magnetic/optical media. For example, the computer readable media may be a hard disk, a floppy disk, a magnetic tape, CD-ROM, Digital Versatile Disk (DVD), Random Access Memory (RAM), or Read Only Memory (ROM) as well as other types of other media known to those skilled in the art.

[0256] Embodiments of the invention include systems (e.g., internet based systems), particularly computer systems which store and manipulate the sequence information described herein. One example of a computer system 100 is illustrated in block diagram form in Figure 1. As used herein, "a computer system" refers to the hardware components, software components, and data storage components used to analyze a nucleotide sequence of a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide sequence as set forth in the Group B amino acid sequences. The computer system 100 typically includes a processor for processing, accessing and manipulating the sequence data. The processor 105 can be any well-known

type of central processing unit, such as, for example, the Pentium III from Intel Corporation, or similar processor from Sun, Motorola, Compaq, AMD or International Business Machines.

[0257] Typically the computer system 100 is a general purpose system that comprises the processor 105 and one or more internal data storage components 110 for storing data, and one or more data retrieving devices for retrieving the data stored on the data storage components. A skilled artisan can readily appreciate that any one of the currently available computer systems are suitable.

[0258] In one particular embodiment, the computer system 100 includes a processor 105 connected to a bus which is connected to a main memory 115 (preferably implemented as RAM) and one or more internal data storage devices 110, such as a hard drive and/or other computer readable media having data recorded thereon. In some embodiments, the computer system 100 further includes one or more data retrieving device 118 for reading the data stored on the internal data storage devices 110.

**[0259]** The data retrieving device 118 may represent, for example, a floppy disk drive, a compact disk drive, a magnetic tape drive, or a modem capable of connection to a remote data storage system (e.g., via the internet) etc. In some embodiments, the internal data storage device 110 is a removable computer readable medium such as a floppy disk, a compact disk, a magnetic tape, etc. containing control logic and/or data recorded thereon. The computer system 100 may advantageously include or be programmed by appropriate software for reading the control logic and/or the data from the data storage component once inserted in the data retrieving device.

[0260] The computer system 100 includes a display 120 which is used to display output to a computer user. It should also be noted that the computer system 100 can be linked to other computer systems 125a-c in a network or wide area network to provide centralized access to the computer system 100.

**[0261]** Software for accessing and processing the nucleotide sequences of a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide sequence as set forth in Group B amino acid sequences,



and sequences substantially identical thereto, (such as search tools, compare tools, and modeling tools etc.) may reside in main memory 115 during execution.

[0262] In some embodiments, the computer system 100 may further comprise a sequence comparison algorithm for comparing a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto, stored on a computer readable medium to a reference nucleotide or polypeptide sequence(s) stored on a computer readable medium. A "sequence comparison algorithm" refers to one or more programs which are implemented (locally or remotely) on the computer system 100 to compare a nucleotide sequence with other nucleotide sequences and/or compounds stored within a data storage means. For example, the sequence comparison algorithm may compare the nucleotide sequences of a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto, stored on a computer readable medium to reference sequences stored on a computer readable medium to identify homologies or structural motifs. Various sequence comparison programs identified elsewhere in this patent specification are particularly contemplated for use in this aspect of the invention. Protein and/or nucleic acid sequence homologies may be evaluated using any of the variety of sequence comparison algorithms and programs known in the art. Such algorithms and programs include, but are by no means limited to, TBLASTN, BLASTP, FASTA, TFASTA, and CLUSTALW (Pearson and Lipman, Proc. Natl. Acad. Sci. USA 85(8):2444-2448, 1988; Altschul et al., J. Mol. Biol. 215(3):403-410, 1990; Thompson et al., Nucleic Acids Res. 22(2):4673-4680, 1994; Higgins et al., Methods Enzymol. 266:383-402, 1996; Altschul et al., J. Mol. Biol. 215(3):403-410, 1990; Altschul et al., Nature Genetics 3:266-272, 1993).

[0263] Homology or identity is often measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Such software matches similar sequences by assigning degrees of homology to various deletions, substitutions and other modifications. The terms "homology" and "identity" in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or

subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same when compared and aligned for maximum correspondence over a comparison window or designated region as measured using any number of sequence comparison algorithms or by manual alignment and visual inspection.

**[0264]** For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

**[0265]** A “comparison window”, as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequence for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2:482, 1981, by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol. 48:443, 1970, by the search for similarity method of Pearson & Lipman, Proc. Nat'l. Acad. Sci. USA 85:2444, 1988, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection. Other algorithms for determining homology or identity include, for example, in addition to a BLAST program (Basic Local Alignment Search Tool at the National Center for Biological Information), ALIGN, AMAS (Analysis of Multiply Aligned Sequences), AMPS (Protein Multiple Sequence Alignment), ASSET (Aligned Segment Statistical Evaluation Tool), BANDS, BESTSCOR, BIOSCAN (Biological Sequence Comparative Analysis Node), BLIMPS (BLocks IMProved Searcher), FASTA, Intervals & Points, BMB, CLUSTAL V, CLUSTAL W, CONSENSUS, LCONSENSUS, WCONSENSUS, Smith-Waterman algorithm, DARWIN, Las Vegas algorithm, FNAT (Forced Nucleotide Alignment Tool),

Framealign, Framesearch, DYNAMIC, FILTER, FSAP (Fristensky Sequence Analysis Package), GAP (Global Alignment Program), GENAL, GIBBS, GenQuest, ISSC (Sensitive Sequence Comparison), LALIGN (Local Sequence Alignment), LCP (Local Content Program), MACAW (Multiple Alignment Construction & Analysis Workbench), MAP (Multiple Alignment Program), MBLKP, MBLKN, PIMA (Pattern-Induced Multi-sequence Alignment), SAGA (Sequence Alignment by Genetic Algorithm) and WHAT-IF. Such alignment programs can also be used to screen genome databases to identify polynucleotide sequences having substantially identical sequences. A number of genome databases are available, for example, a substantial portion of the human genome is available as part of the Human Genome Sequencing Project (J. Roach, [http://weber.u.Washington.edu/~roach/human\\_genome\\_progress\\_2.html](http://weber.u.Washington.edu/~roach/human_genome_progress_2.html)) (Gibbs, 1995). At least twenty-one other genomes have already been sequenced, including, for example, M. genitalium (Fraser et al., 1995), M. jannaschii (Bult et al., 1996), H. influenzae (Fleischmann et al., 1995), E. coli (Blattner et al., 1997), and yeast (S. cerevisiae) (Mewes et al., 1997), and D. melanogaster (Adams et al., 2000). Significant progress has also been made in sequencing the genomes of model organism, such as mouse, C. elegans, and Arabadopsis sp. Several databases containing genomic information annotated with some functional information are maintained by different organization, and are accessible via the internet, for example, <http://www.tigr.org/tdb>; <http://www.genetics.wisc.edu>; <http://genome-www.stanford.edu/~ball>; <http://hiv-web.lanl.gov>; <http://www.ncbi.nlm.nih.gov>; <http://www.ebi.ac.uk>; <http://Pasteur.fr/other/biology>; and <http://www.genome.wi.mit.edu>.

[0266] One example of a useful algorithm is BLAST and BLAST 2.0 algorithms, which are described in Altschul et al., Nuc. Acids Res. 25:3389-3402, 1997, and Altschul et al., J. Mol. Biol. 215:403-410, 1990, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al., supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both

directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always  $>0$ ). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectations (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA 89:10915, 1989) alignments (B) of 50, expectation (E) of 10, M=5, N= -4, and a comparison of both strands.

[0267] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, Proc. Natl. Acad. Sci. USA 90:5873, 1993). One measure of similarity provided by BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a references sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

[0268] In one embodiment, protein and nucleic acid sequence homologies are evaluated using the Basic Local Alignment Search Tool ("BLAST") In particular, five specific BLAST programs are used to perform the following task:

[0269] (1) BLASTP and BLAST3 compare an amino acid query sequence against a protein sequence database;

[0270] (2) BLASTN compares a nucleotide query sequence against a nucleotide sequence database;

[0271] (3) BLASTX compares the six-frame conceptual translation products of a query nucleotide sequence (both strands) against a protein sequence database;

[0272] (4) TBLASTN compares a query protein sequence against a nucleotide sequence database translated in all six reading frames (both strands); and

[0273] (5) TBLASTX compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database.

[0274] The BLAST programs identify homologous sequences by identifying similar segments, which are referred to herein as "high-scoring segment pairs," between a query amino or nucleic acid sequence and a test sequence which is preferably obtained from a protein or nucleic acid sequence database. High-scoring segment pairs are preferably identified (i.e., aligned) by means of a scoring matrix, many of which are known in the art. Preferably, the scoring matrix used is the BLOSUM62 matrix (Gonnet et al., Science 256:1443-1445, 1992; Henikoff and Henikoff, Proteins 17:49-61, 1993). Less preferably, the PAM or PAM250 matrices may also be used (see, e.g., Schwartz and Dayhoff, eds., 1978, Matrices for Detecting Distance Relationships: Atlas of Protein Sequence and Structure, Washington: National Biomedical Research Foundation). BLAST programs are accessible through the U.S. National Library of Medicine, e.g., at [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov).

[0275] The parameters used with the above algorithms may be adapted depending on the sequence length and degree of homology studied. In some embodiments, the parameters may be the default parameters used by the algorithms in the absence of instructions from the user.

[0276] Figure 2 is a flow diagram illustrating one embodiment of a process 200 for comparing a new nucleotide or protein sequence with a database of sequences in order to determine the homology levels between the new sequence and the sequences in the database. The database of sequences can be a private database stored within the computer system 100, or a public database such as GENBANK that is available through the Internet.

[0277] The process 200 begins at a start state 201 and then moves to a state 202 wherein the new sequence to be compared is stored to a memory in a computer system 100.

As discussed above, the memory could be any type of memory, including RAM or an internal storage device.

[0278] The process 200 then moves to a state 204 wherein a database of sequences is opened for analysis and comparison. The process 200 then moves to a state 206 wherein the first sequence stored in the database is read into a memory on the computer. A comparison is then performed at a state 210 to determine if the first sequence is the same as the second sequence. It is important to note that this step is not limited to performing an exact comparison between the new sequence and the first sequence in the database. Well-known methods are known to those of skill in the art for comparing two nucleotide or protein sequences, even if they are not identical. For example, gaps can be introduced into one sequence in order to raise the homology level between the two tested sequences. The parameters that control whether gaps or other features are introduced into a sequence during comparison are normally entered by the user of the computer system.

[0279] Once a comparison of the two sequences has been performed at the state 210, a determination is made at a decision state 210 whether the two sequences are the same. Of course, the term "same" is not limited to sequences that are absolutely identical. Sequences that are within the homology parameters entered by the user will be marked as "same" in the process 200.

[0280] If a determination is made that the two sequences are the same, the process 200 moves to a state 214 wherein the name of the sequence from the database is displayed to the user. This state notifies the user that the sequence with the displayed name fulfills the homology constraints that were entered. Once the name of the stored sequence is displayed to the user, the process 200 moves to a decision state 218 wherein a determination is made whether more sequences exist in the database. If no more sequences exist in the database, then the process 200 terminates at an end state 220. However, if more sequences do exist in the database, then the process 200 moves to a state 224 wherein a pointer is moved to the next sequence in the database so that it can be compared to the new sequence. In this manner, the new sequence is aligned and compared with every sequence in the database.

[0281] It should be noted that if a determination had been made at the decision state 212 that the sequences were not homologous, then the process 200 would move immediately

to the decision state 218 in order to determine if any other sequences were available in the database for comparison.

[0282] Accordingly, one aspect of the invention is a computer system comprising a processor, a data storage device having stored thereon a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto, a data storage device having retrievably stored thereon reference nucleotide sequences or polypeptide sequences to be compared to a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto, and a sequence comparer for conducting the comparison. The sequence comparer may indicate a homology level between the sequences compared or identify structural motifs in the above described nucleic acid code of Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto, or it may identify structural motifs in sequences which are compared to these nucleic acid codes and polypeptide codes. In some embodiments, the data storage device may have stored thereon the sequences of at least 2, 5, 10, 15, 20, 25, 30 or 40 or more of the nucleic acid sequences as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or the polypeptide sequences as set forth in Group B amino acid sequences, and sequences substantially identical thereto.

[0283] Another aspect of the invention is a method for determining the level of homology between a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto, and a reference nucleotide sequence. The method including reading the nucleic acid code or the polypeptide code and the reference nucleotide or polypeptide sequence through the use of a computer program which determines homology levels and determining homology between the nucleic acid code or polypeptide code and the reference nucleotide or polypeptide sequence with the computer program. The computer program may be any of a number of computer programs for determining homology levels, including those specifically enumerated herein, (e.g.,

BLAST2N with the default parameters or with any modified parameters). The method may be implemented using the computer systems described above. The method may also be performed by reading at least 2, 5, 10, 15, 20, 25, 30 or 40 or more of the above described nucleic acid sequences as set forth in the Group A nucleic acid sequences, or the polypeptide sequences as set forth in the Group B amino acid sequences through use of the computer program and determining homology between the nucleic acid codes or polypeptide codes and reference nucleotide sequences or polypeptide sequences.

[0284] Figure 3 is a flow diagram illustrating one embodiment of a process 250 in a computer for determining whether two sequences are homologous. The process 250 begins at a start state 252 and then moves to a state 254 wherein a first sequence to be compared is stored to a memory. The second sequence to be compared is then stored to a memory at a state 256. The process 250 then moves to a state 260 wherein the first character in the first sequence is read and then to a state 262 wherein the first character of the second sequence is read. It should be understood that if the sequence is a nucleotide sequence, then the character would normally be either A, T, C, G or U. If the sequence is a protein sequence, then it is preferably in the single letter amino acid code so that the first and sequence sequences can be easily compared.

[0285] A determination is then made at a decision state 264 whether the two characters are the same. If they are the same, then the process 250 moves to a state 268 wherein the next characters in the first and second sequences are read. A determination is then made whether the next characters are the same. If they are, then the process 250 continues this loop until two characters are not the same. If a determination is made that the next two characters are not the same, the process 250 moves to a decision state 274 to determine whether there are any more characters either sequence to read.

[0286] If there are not any more characters to read, then the process 250 moves to a state 276 wherein the level of homology between the first and second sequences is displayed to the user. The level of homology is determined by calculating the proportion of characters between the sequences that were the same out of the total number of sequences in the first sequence. Thus, if every character in a first 100 nucleotide sequence aligned with a every character in a second sequence, the homology level would be 100%.



[0287] Alternatively, the computer program may be a computer program which compares the nucleotide sequences of a nucleic acid sequence as set forth in the invention, to one or more reference nucleotide sequences in order to determine whether the nucleic acid code of Group A nucleic acid sequences, and sequences substantially identical thereto, differs from a reference nucleic acid sequence at one or more positions. Optionally such a program records the length and identity of inserted, deleted or substituted nucleotides with respect to the sequence of either the reference polynucleotide or a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto. In one embodiment, the computer program may be a program which determines whether a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, contains a single nucleotide polymorphism (SNP) with respect to a reference nucleotide sequence.

[0288] Accordingly, another aspect of the invention is a method for determining whether a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, differs at one or more nucleotides from a reference nucleotide sequence comprising the steps of reading the nucleic acid code and the reference nucleotide sequence through use of a computer program which identifies differences between nucleic acid sequences and identifying differences between the nucleic acid code and the reference nucleotide sequence with the computer program. In some embodiments, the computer program is a program which identifies single nucleotide polymorphisms. The method may be implemented by the computer systems described above and the method illustrated in Figure 3. The method may also be performed by reading at least 2, 5, 10, 15, 20, 25, 30, or 40 or more of the nucleic acid sequences as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, and the reference nucleotide sequences through the use of the computer program and identifying differences between the nucleic acid codes and the reference nucleotide sequences with the computer program.

[0289] In other embodiments the computer based system may further comprise an identifier for identifying features within a nucleic acid sequence as set forth in the Group A nucleic acid sequences or a polypeptide sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto.

[0290] An "identifier" refers to one or more programs which identifies certain features within a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto. In one embodiment, the identifier may comprise a program which identifies an open reading frame in a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto.

[0291] Figure 5 is a flow diagram illustrating one embodiment of an identifier process 300 for detecting the presence of a feature in a sequence. The process 300 begins at a start state 302 and then moves to a state 304 wherein a first sequence that is to be checked for features is stored to a memory 115 in the computer system 100. The process 300 then moves to a state 306 wherein a database of sequence features is opened. Such a database would include a list of each feature's attributes along with the name of the feature. For example, a feature name could be "Initiation Codon" and the attribute would be "ATG". Another example would be the feature name "TAATAA Box" and the feature attribute would be "TAATAA". An example of such a database is produced by the University of Wisconsin Genetics Computer Group ([www.gcg.com](http://www.gcg.com)). Alternatively, the features may be structural polypeptide motifs such as alpha helices, beta sheets, or functional polypeptide motifs such as enzymatic active sites, helix-turn-helix motifs or other motifs known to those skilled in the art.

[0292] Once the database of features is opened at the state 306, the process 300 moves to a state 308 wherein the first feature is read from the database. A comparison of the attribute of the first feature with the first sequence is then made at a state 310. A determination is then made at a decision state 316 whether the attribute of the feature was found in the first sequence. If the attribute was found, then the process 300 moves to a state 318 wherein the name of the found feature is displayed to the user.

[0293] The process 300 then moves to a decision state 320 wherein a determination is made whether more features exist in the database. If no more features do exist, then the process 300 terminates at an end state 324. However, if more features do exist in the database, then the process 300 reads the next sequence feature at a state 326 and loops back

to the state 310 wherein the attribute of the next feature is compared against the first sequence.

[0294] It should be noted, that if the feature attribute is not found in the first sequence at the decision state 316, the process 300 moves directly to the decision state 320 in order to determine if any more features exist in the database.

[0295] Accordingly, another aspect of the invention is a method of identifying a feature within a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto, comprising reading the nucleic acid code(s) or polypeptide code(s) through the use of a computer program which identifies features therein and identifying features within the nucleic acid code(s) with the computer program. In one embodiment, computer program comprises a computer program which identifies open reading frames. The method may be performed by reading a single sequence or at least 2, 5, 10, 15, 20, 25, 30, or 40 of the nucleic acid sequences as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or the polypeptide sequences as set forth in Group B amino acid sequences, and sequences substantially identical thereto, through the use of the computer program and identifying features within the nucleic acid codes or polypeptide codes with the computer program.

[0296] A nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto, may be stored and manipulated in a variety of data processor programs in a variety of formats. For example, a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto, may be stored as text in a word processing file, such as MicrosoftWORD or WORDPERFECT or as an ASCII file in a variety of database programs familiar to those of skill in the art, such as DB2, SYBASE, or ORACLE. In addition, many computer programs and databases may be used as sequence comparison algorithms, identifiers, or sources of reference nucleotide sequences or polypeptide sequences to be compared to a nucleic acid sequence as set forth in Group A

nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto. The following list is intended not to limit the invention but to provide guidance to programs and databases which are useful with the nucleic acid sequences as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or the polypeptide sequences as set forth in Group B amino acid sequences, and sequences substantially identical thereto.

[0297] The programs and databases which may be used include, but are not limited to: MacPattern (EMBL), DiscoveryBase (Molecular Applications Group), GeneMine (Molecular Applications Group), Look (Molecular Applications Group), MacLook (Molecular Applications Group), BLAST and BLAST2 (NCBI), BLASTN and BLASTX (Altschul et al, J. Mol. Biol. 215: 403, 1990), FASTA (Pearson and Lipman, Proc. Natl. Acad. Sci. USA, 85: 2444, 1988), FASTDB (Brutlag et al. Comp. App. Biosci. 6:237-245, 1990), Catalyst (Molecular Simulations Inc.), Catalyst/SHAPE (Molecular Simulations Inc.), Cerius2.DBAccess (Molecular Simulations Inc.), HypoGen (Molecular Simulations Inc.), Insight II, (Molecular Simulations Inc.), Discover (Molecular Simulations Inc.), CHARMm (Molecular Simulations Inc.), Felix (Molecular Simulations Inc.), DelPhi, (Molecular Simulations Inc.), QuanteMM, (Molecular Simulations Inc.), Homology (Molecular Simulations Inc.), Modeler (Molecular Simulations Inc.), ISIS (Molecular Simulations Inc.), Quanta/Protein Design (Molecular Simulations Inc.), WebLab (Molecular Simulations Inc.), WebLab Diversity Explorer (Molecular Simulations Inc.), Gene Explorer (Molecular Simulations Inc.), SeqFold (Molecular Simulations Inc.), the MDL Available Chemicals Directory database, the MDL Drug Data Report data base, the Comprehensive Medicinal Chemistry database, Derwent's World Drug Index database, the BioByteMasterFile database, the Genbank database, and the Genseqn database. Many other programs and data bases would be apparent to one of skill in the art given the present disclosure.

[0298] Motifs which may be detected using the above programs include sequences encoding leucine zippers, helix-turn-helix motifs, glycosylation sites, ubiquitination sites, alpha helices, and beta sheets, signal sequences encoding signal peptides which direct the secretion of the encoded proteins, sequences implicated in transcription regulation such as

homeoboxes, acidic stretches, enzymatic active sites, substrate binding sites, and enzymatic cleavage sites.

[0299] The present invention exploits the unique catalytic properties of enzymes. Whereas the use of biocatalysts (i.e., purified or crude enzymes, non-living or living cells) in chemical transformations normally requires the identification of a particular biocatalyst that reacts with a specific starting compound, the present invention uses selected biocatalysts and reaction conditions that are specific for functional groups that are present in many starting compounds, such as small molecules. Each biocatalyst is specific for one functional group, or several related functional groups, and can react with many starting compounds containing this functional group.

[0300] The biocatalytic reactions produce a population of derivatives from a single starting compound. These derivatives can be subjected to another round of biocatalytic reactions to produce a second population of derivative compounds. Thousands of variations of the original small molecule or compound can be produced with each iteration of biocatalytic derivatization.

[0301] Enzymes react at specific sites of a starting compound without affecting the rest of the molecule, a process which is very difficult to achieve using traditional chemical methods. This high degree of biocatalytic specificity provides the means to identify a single active compound within the library. The library is characterized by the series of biocatalytic reactions used to produce it, a so called "biosynthetic history". Screening the library for biological activities and tracing the biosynthetic history identifies the specific reaction sequence producing the active compound. The reaction sequence is repeated and the structure of the synthesized compound determined. This mode of identification, unlike other synthesis and screening approaches, does not require immobilization technologies, and compounds can be synthesized and tested free in solution using virtually any type of screening assay. It is important to note, that the high degree of specificity of enzyme reactions on functional groups allows for the "tracking" of specific enzymatic reactions that make up the biocatalytically produced library.

[0302] Many of the procedural steps are performed using robotic automation enabling the execution of many thousands of biocatalytic reactions and screening assays per day as

well as ensuring a high level of accuracy and reproducibility. As a result, a library of derivative compounds can be produced in a matter of weeks which would take years to produce using current chemical methods.

[0303] In a particular embodiment, the invention provides a method for modifying small molecules, comprising contacting a polypeptide encoded by a polynucleotide described herein or enzymatically active fragments thereof with a small molecule to produce a modified small molecule. A library of modified small molecules is tested to determine if a modified small molecule is present within the library which exhibits a desired activity. A specific biocatalytic reaction which produces the modified small molecule of desired activity is identified by systematically eliminating each of the biocatalytic reactions used to produce a portion of the library, and then testing the small molecules produced in the portion of the library for the presence or absence of the modified small molecule with the desired activity. The specific biocatalytic reactions which produce the modified small molecule of desired activity is optionally repeated. The biocatalytic reactions are conducted with a group of biocatalysts that react with distinct structural moieties found within the structure of a small molecule, each biocatalyst is specific for one structural moiety or a group of related structural moieties; and each biocatalyst reacts with many different small molecules which contain the distinct structural moiety.

[0304] In another embodiment, the novel alkaline amylases of the invention were identified by screening for both activity at high pH and identification of amylases with stability in an automatic dish wash (ADW) formulation. Comparisons were made to the amylase derived from *Bacillus licheniformis*. A study of the dependence of hydrolysis on pH showed that the majority of the alkaline amylases of the invention have a pH optima of 7 or less, the exception is clone B with a pH optima of approximately 8. The alkaline amylases of the invention retain activity in ADW formulations, though clone B is sensitive to high temperatures. Preferably, when used in ADW products, the alkaline amylase of the invention will function at a pH 10-11 and at 45-60°C.

[0305] The invention will be further described with reference to the following examples; however, it is to be understood that the invention is not limited to such examples.

## EXAMPLES

### EXAMPLE 1

#### IDENTIFICATION AND CHARACTERIZATION OF THERMOSTABLE $\alpha$ -AMYLASES

The present example shows the identification of novel acid amylases. The screening program was carried out under neutral and low pH conditions. DNA libraries generated from low pH samples were targeted for discovery. This effort afforded the discovery of hundreds of clones having the ability to degrade starch. DNA sequence and bioinformatic analyses classified many of these genes as previously unidentified amylases.

#### Biochemical studies

Biochemical analysis of the amylase genomic clones showed that many had pH optima of less than pH 6. Lysates of these genomic clones were tested for thermal tolerance by incubation at 70°C, 80°C, 90°C or 100°C for 10 minutes and measurement of residual activity at pH 4.5. Those clones retaining >50% activity after heat treatment at 80°C were chosen for further analysis. These clones were incubated at 90°C for 10 minutes at pH 6.0 and 4.5 and tested for residual activity at pH 4.5 (Figure 1). A number of clones retained >40% of their activity following this treatment. For comparative purposes, residual activity of an evolved amylase, clone c, was equivalent to the best of the second-generation enzymes; the specific activity of clone c was greater.

Thermal activity of the clones with residual activity after heat treatment at 90°C at pH 4.5 was measured at room temperature, 70°C and 90°C at pH 4.5. Table 1 shows that the hydrolysis rates of SEQ ID NO.: 87 (*B. stearothermophilus* amylase) and SEQ ID NO. 113 (*B. licheniformis* amylase) decrease at higher temperatures, whereas the rate for SEQ ID NO.:125 continues to increase as the temperature is raised to 70°C and only reduces by around 50% at 90°C.

**Candidate evaluation**

Based on residual activity at pH 4.5 after a 90 °C heat treatment, specific activity and rate of starch hydrolysis at 90 °C when compared with *B.licheniformis* amylase, SEQ ID NO.:125 is compared with the evolved amylase clone c in a starch liquefaction assay.

	Room temperature	70°C	90°
SEQ ID NO.:87 <sup>1</sup>	1.25	1.43	0.33
SEQ ID NO.: 113 <sup>2</sup>	3.3	1.9	0.39
SEQ ID NO.: 125	1.9	47	19

Table 1. Rates of dye labeled starch hydrolysis (relative fluorescence units/s) of three genomic clones at pH 4.5 and 3 different temperatures. <sup>1</sup>*B. stearotherophilus* amylase, <sup>2</sup>*B. licheniformis* amylase

**EXAMPLE 2****THERMOSTABLE AMYLASES ACTIVE AT ALKALINE pH**

The initial focus of this example was the evaluation of an existing panel of amylases in an commercial automatic dish wash (ADW) formulation. This effort identified two candidates: one with activity at high pH (SEQ ID NO.:115) and another with stability in the ADW formulation (SEQ ID NO.:207). Studies also included the identification of high pH amylases. This effort afforded the discovery of hundreds of clones having the ability to degrade starch. DNA sequence and bioinformatics analyses classified many of these genes as previously unidentified amylases. The remaining open reading frames were neopullulanases, amylopullulanases and amylomaltases. Extensive biochemical and applications studies showed that 3 candidates: clone B, SEQ ID NO.:147 and SEQ ID NO.:139) have high specific activity at pH10, but unfortunately lack stability in the ADW formulation. In summary, a panel of novel amylases each having desirable phenotypes for the ADW application has been identified.



### Biochemical studies

Biochemical analysis of the amylase genomic clones showed that many of them hydrolyzed starch at pH 10 and 50°C. To produce sufficient quantities of enzyme for further biochemical and applications testing, the amylase open reading frames of the 40 most active genomic clones were subcloned into expression vectors. This effort included making 2 constructs for those clones containing a putative signal sequence and establishing the growth and induction conditions for each subclone (plus and minus the amylase signal peptide).

Soluble, active protein was successfully purified to homogeneity from 34 subclones and specific activity (units/mg, where 1 unit =  $\mu\text{mol}$  reducing sugars/min) was measured at pH 8 and pH 10 (40°C and 50°C) using 2% starch in buffer. The amylase from *Bacillus licheniformis* (SEQ ID NO.:113) was chosen as the benchmark for these studies. Specific activity was determined by removing samples at various time points during a 30 minute reaction and analyzing for reducing sugars. The initial rate was determined by fitting the progress curves to a linear equation. A comparison of the top candidates is shown in Table 2.

A study to determine the dependence of hydrolysis rate on pH showed that only clone B is an "alkaline amylase" with a pH optimum of approximately 8; all others had pH optima of 7 or less. Nevertheless, it is clear that the panel of hits included several lead amylases with appreciable activity at pH 10 and 50°C.

**Table 2. Specific activities (U/mg pure enzyme) of amylases**

Enzyme	Specific activity pH 8, 40°C	Specific activity pH 10, 50°C
Clone B	682	20
SEQ ID NO.:139	430	33
SEQ ID NO.:127	250	47
SEQ ID NO.:137	230	3
SEQ ID NO.:113 ( <i>B. licheniformis</i> )	228	27
SEQ ID NO.:205	163	4
Remainder	<40	

### Stability

Stability in the presence of the ADW formulation was measured for each of the 3 top candidates identified via biochemical analysis. The benchmark for these studies was a commercial enzyme in the formulation matrix. Figure 13 illustrates the residual activity (measured at pH 8 and 50°C) after a 30 minute incubation at 50°C in the presence of various components of the ADW formulation; pH 8, pH 10.8, ADW solution (with bleach) and ADW solution (without bleach). The measured activity after the incubation is expressed as a percentage of the original activity. The data show that clone B was very sensitive to high temperature, whereas the other amylases were less affected. When the enzymes were incubated at high pH and temperature, the commercial enzyme SEQ ID NO.: 139 became less stable; however, SEQ ID NO.: 127 retained full activity. The apparently anomalous behavior of SEQ ID NO.: 127 after pH 10 incubation vs pH 8 was observed in repeated trials.

When amylase activity on dye-labeled starch is measured in the ADW matrix at 50°C, the commercial amylase exhibits roughly 5% of its activity at pH 8. In the same assay, clone B, SEQ ID NO.: 139 and SEQ ID NO.: 127 exhibit <2% of their original activity measured at pH 8.

#### Wash tests

Wash tests using starch coated slides were carried out to gauge the performance of each of the purified enzymes as compared to the commercial amylase. The spaghetti starch coated slides were prepared according to protocol. Two pre-weighed starch coated slides were placed back to back in a 50 mL conical tube and 25 mL of ADW solution, +/- enzyme were added per tube. The tubes were incubated for 20 minutes at 50°C with gentle rotation on a vertical carousel. Following the incubation period, the slides were immediately rinsed in water and oven dried overnight. All trials were run in duplicate and the commercial enzyme was run as a positive control. The results (Figure 6) of these experiments are expressed as net % starch removed, e.g. % of starch removed in ADW with enzyme, *minus* the % of starch removed in ADW alone.

### EXAMPLE 3

#### GENE OPTIMIZATION

The properties of enzymes may be improved by various evolution strategies, including GeneSiteSaturationMutagenesis (GSSM<sup>TM</sup>) and GeneReassembly<sup>TM</sup>. (Diversa Corporation, San Diego, CA). Such techniques will be applied to the discovered amylase genes in order to generate pools of variants that can be screened for improved performance.

Parental molecules for evolution will be one or all of the following: SEQ ID NO.: 113, SEQ ID NO.: 139, SEQ ID NO.:115 and SEQ ID NO.: 127 (a truncated form of SEQ ID NO.: 127).

A high throughput screen has been developed to assess enzyme performance in the presence of ADW performance. Development of a HTS is of paramount importance in any evolution program The HTS is automated and has showed consistent results for the parental amylases (Figure 7). Parental amylases have measurable activity in the ADW formulation, however highly reduced relative to pH 8 activity.

#### **EXAMPLE 4**

##### **CHARACTERIZATION OF $\alpha$ -AMYLASES HAVING ACTIVITY AT ALKALINE pH**

Amylases of the invention having activity at alkaline pH were characterized further. Kinetics on 2% starch at pH 8 and 10 (40°C and 50°C) have been performed.

Table 4:

<u>Clones, specific activities</u>	<u>pH 8, 40°C</u>	<u>pH 10, 50°C</u>
SEQ ID NO.: 113 ( <i>B. licheniformis</i> )	228 units/mg	27 units/mg
Clone B	682 units/mg	31 units/mg
SEQ ID NO.: 139	430 units/mg	33 units/mg
SEQ ID NO.: 127	540 units/mg	50 units/mg
control 0GL5 ( <i>E. coli</i> )	1.8 units/mg	0 units/mg

1 unit of activity is defined as release of 1  $\mu$ mol reducing sugars per minute.

#### **EXAMPLE 5**

##### **AMYLASE ACTIVITY ASSAY: BCA REDUCING ENDS ASSAY**

Amylase activity of clones of interest was determined using the following methodology.

1. Prepare 2 substrate solutions, as follows:

- a) 2% soluble starch (potato) pH 8 solution by dissolving 2 gm potato starch in 100 ml 100 mM sodium phosphate pH 8).
- b) 2% soluble starch (potato) pH 10 solution by dissolving 2 gm potato starch in 100 ml 100 mM sodium carbonate.

Heat both solutions in a boiling water bath, while mixing, for 30-40 minutes until starch dissolves.

2. Prepare Solution A from 64 mg/ml sodium carbonate monohydrate, 24 mg/ml sodium bicarbonate and 1.95 mg/ml BCA (4,4'-dicarboxy-2,2'-biquinoline disodium salt (Sigma Chemical cat # D-8284). Added above to dH<sub>2</sub>O.
3. Prepare solution B by combining 1.24 mg/ml cupric sulfate pentahydrate and 1.26 mg/ml L-serine. Add mixture to to dH<sub>2</sub>O.
4. Prepare a working reagent of a 1:1 ration of solutions A and B.
5. Prepare a Maltose standard solution of 10 mM Maltose in dH<sub>2</sub>O, where the 10 mM maltose is combined in 2% soluble starch at desired pH to a final concentration of 0, 100, 200, 300, 400, 600  $\mu$ M. The standard curve will be generated for each set of time-points. Since the curve is determined by adding 10 ul of the standards to the working reagent it works out to 0, 1, 2, 3, 4, 6 nmole maltose.
6. Aliquot 1 ml of substrate solution into microcentrifuge tubes, equilibrate to desired temperature (5 min) in heat block or heated water bath. Add 50 ul of enzyme solution to the inside of the tube lid.
7. While solution is equilibrating mix 5 ml of both solution A & B. Aliquot 100 ul to 96 well PCR plate. Set plate on ice.
8. After 5 minute temperature equilibration, close lid on tubes, invert and vortex 3 times. Immediately aliquot 10 ul into plate as t=0 (zero time point). Leave enzyme mixture in heat block and aliquot 10 ul at each desired time point (e.g. 0, 5, 10, 15, 20, 30 minutes).
9. Ensure that 12 wells are left empty (only working reagent aliquotted) for the addition of 10 ul of standards, for the standard curve.
10. When all time points are collected and standards are added, cover plate and heated to 80° C for 35 min. Cool plate on ice for 10 min. Add 100 ul H<sub>2</sub>O to all wells. Mix and aliquot 100 ul into flat bottomed 96-well plate and read absorbance at 560 nm.
11. Zero each sample's time points against its own t=0 (subtract the average t=0 A<sub>560</sub> value from other average A<sub>560</sub> values). Convert the A<sub>560</sub><sub>(experimental)</sub> to umole (Divide A<sub>560</sub><sub>(experimental)</sub> by

the slope of the standard curve (A560/umole). Generate a slope of the time points and the umole (in umole/min), multiply by 100 (as the umole value only accounts for the 10 ul used in the assay, not the amount made in the 1ml rxn). To get the specific activity divide the slope (in umole/min) by the mg of protein. All points should be done at a minimum in duplicate with three being best. An example standard curve is set forth in Figure 11.

Table 5: Sample data:

<u>Clone</u>	<u>Dilution</u>	<u>Minutes</u>	<u>A560-1</u>	<u>A560-2</u>	<u>Avg A 560</u>	<u>Zeroed A 560</u>	(A560exp/std slope)
							<u>umole</u>
ENZ	50	0	0.1711	0.1736	0.17235	0	0.0000
		5	0.2104	0.2165	0.21345	0.0411	0.0005
		10	0.2492	0.2481	0.24865	0.0763	0.0009
		15	0.2984	0.2882	0.2933	0.12095	0.0014
		20	0.3355	0.3409	0.3382	0.16585	0.0020
		30	0.3942	0.3805	0.38735	0.215	0.0026
		40	0.4501	0.4412	0.44565	0.2733	0.0033

Activity = 0.008646 umole/min

Divide protein concentration (mg/ml) by any dilution to get mg used in assay.

Divide the above slope by mg used in assay to get specific activity

Specific Activity = 24.93 umole/min/mg

(See for example, Dominic W.S. Wong, Sarah B. Batt, and George H. Robertson (2000).

Microassay for rapid screening of alpha-amylase activity. J. Agric.Food Chem. 48,4540-4543 and

Jeffrey D. Fox and John F. Robyt, (1991). Minuturization of three carbohydrate analyses using a

micro sample plate reader. Anal. Biochem. 195, 93-96, herein incorporated by reference).

#### EXAMPLE 6

#### SCREENING FOR $\alpha$ -AMYLASE ACTIVITY

Amylase activity of clones can be assessed by a number of methods known in the art. The following is the general methodology that was used in the present invention. The number of plaques screened, per plate, should be approximately 10,000 pfu's. For each DNA library: at least 50,000 plaques per isolated library and 200,000 plaques per non-isolated library should be screened depending upon the pfu titer for the  $\lambda$  Zap Express amplified lysate.

#### Titer determination of Lambda Library

- 1)  $\mu$ L of Lambda Zap Express amplified library stock added to 600 $\mu$ L *E. coli* MRF' cells (OD<sub>600</sub>=1.0). To dilute MRF' stock, 10mM MgSO<sub>4</sub> is used.
- 2) Incubate at 37 °C for 15 minutes.
- 3) Transfer suspension to 5-6mL of NZY top agar at 50 °C and gently mix.
- 4) Immediately pour agar solution onto large (150mm) NZY media plate.
- 5) Allow top agar to solidify completely (approximately 30 minutes), then invert plate.
- 6) Incubate the plate at 39 °C for 8-12 hours.
- 7) Number of plaques is approximated. Phage titer determined to give 10,000 pfu/plate. Dilute an aliquot of Library phage with SM buffer if needed.

#### Substrate screening

- 1) Lambda Zap Express (50,000 pfu) from amplified library added to 600 $\mu$ L of *E. coli* MRF' cells (OD<sub>600</sub>=1.0). For non-environment libraries, prepare 4 tubes (50,000 pfu per tube).
- 2) Incubate at 37 °C for 15 minutes.
- 3) While phage/cell suspension are incubating, 1.0mL of red starch substrate (1.2% w/v) is added to 6.0mL NZY top agar at 50 °C and mixed thoroughly. Keep solution at 50°C until needed.
- 4) Transfer 1/5 (10,000 pfu) of the cell suspension to substrate/top agar solution and gently mixed.
- 5) Solution is immediately poured onto large (150mm) NZY media plate.
- 6) Allow top agar to solidify completely (approximately 30 minutes), then invert plate.

- 7) Repeat procedures 4-6 4 times for the rest of the cell suspension (1/5 of the suspension each time).
- 8) Incubate plates at 39 °C for 8-12 hours.
- 9) Plate observed for clearing zones (halos) around plaques.
- 10) Plaques with halos are cored out of agar and transferred to a sterile micro tube. A large bore 200µL pipette tip works well to remove (core) the agar plug containing the desired plaque.
- 11) Phages are re-suspended in 500µL SM buffer. 20µL Chloroform is added to inhibit any further cell growth.
- 12) Pure phage suspension is incubated at room temperature for 4 hours or overnight before next step.

#### Isolation of pure clones

- 1) 10µL of re-suspended phage suspension is added to 500µL of *E. coli* MRF' cells (OD600=1.0).
- 2) Incubate at 37 °C for 15 minutes.
- 3) While phage/cell suspension is incubating, 1mL of red starch substrate (1.2% w/v) is added to 6.0mL NZY top agar at 50 °C and mixed thoroughly. Keep solution at 50 °C until needed.
- 4) Cell suspension is transferred to substrate/top agar solution and gently mixed.
- 5) Solution is immediately poured onto large (150mm) NZY media plate.
- 6) Allow top agar to solidify completely (approximately 30 minutes), then invert plate.
- 7) Plate incubated at 39 °C for 8-12 hours.
- 8) Plate observed for a clearing zone (halo) around a single plaque (pure clone). If a single plaque cannot be isolated, adjust titer and re-plate phage suspension.
- 9) Single plaque with halo is cored out of agar and transferred to a sterile micro tube. A large bore 200µL pipette tip works well to remove (core) the agar plug containing the desired plaque. To amplify the titer, core 5 single active plaques into a micro tube.
- 10) Phages are re-suspended in 500µL SM buffer. 20µL Chloroform is added to inhibit any further cell growth.

- 11) Pure phage suspension is incubated at room temperature for 4 hours or overnight before next step. The pure phage suspension is stored at  $-80^{\circ}\text{C}$  by adding DMSO into the phage suspension (7% v/v).

Excision of pure clone

- 1) 100 $\mu\text{L}$  of pure phage suspension is added to 200 $\mu\text{L}$  *E. coli* MRF' cells (OD600=1.0). To this, 1.0 $\mu\text{L}$  of ExAssist helper phage ( $>1 \times 10^6$  pfu/mL; Stratagene) is added. Use 2059 Falcon tube for excision.
- 2) Suspension is incubated at  $37^{\circ}\text{C}$  for 15 minutes.
- 3) 3.0 mL of 2 x YT media is added to cell suspension.
- 4) Incubate at  $30^{\circ}\text{C}$  for at least 6 hours or overnight while shaking.
- 5) Tube transferred to  $70^{\circ}\text{C}$  for 20 minutes. The phagemid suspension can be stored at  $4^{\circ}\text{C}$  for 1 to 2 months.
- 6) 100  $\mu\text{L}$  of phagemid suspension transferred to a micro tube containing 200 $\mu\text{L}$  of *E. coli* Exp 505 cells (OD600=1.0).
- 7) Suspension incubated at  $37^{\circ}\text{C}$  for 15 minutes.
- 8) 300 $\mu\text{L}$  of SOB is added to the suspension.
- 9) Suspension is incubated at  $37^{\circ}\text{C}$  for 30 to 45 minutes.
- 10) 100 $\mu\text{L}$  of suspension is transferred to a small (90mm) LB media plate containing Kanamycin (LB media with Kanamycin 50 $\mu\text{g/mL}$ ) for Zap Express DNA libraries or Ampicillin (LB media with Kanamycin 100 $\mu\text{g/mL}$ ) for Zap II DNA libraries.
- 11) The rest of suspension is transferred to another small LB media plate.
- 12) Use sterile glass beads to evenly distribute suspension on the plate.
- 13) Plates are incubated at  $30^{\circ}\text{C}$  for 12 to 24 hours.
- 14) Plate observed for colonies.
- 15) Inoculate single colony into LB liquid media containing suitable antibiotic and incubate at  $30^{\circ}\text{C}$  for 12 to 24 hours.
- 16) Glycerol stock can be prepared by adding 80% glycerol into liquid culture (15% v/v) and stored at  $-80^{\circ}\text{C}$ .



#### Activity verification

- 1) 50 $\mu$ L of liquid culture is transferred to a micro tube. Add 500 $\mu$ L of 8% pH7 Amylopectin Azure into the same tube. Prepare 2 tubes for each clone.
- 2) Activity is tested at 50 °C for 3 hours and overnight. Use pH 7 buffer as control.
- 3) Cool the test specimen at ice-water bath for 5 minutes.
- 4) Add 750 $\mu$ L of Ethaanol and mixed thoroughly.
- 5) Centrifuge at 13000 rpm (16000 g's) for 5 minutes.
- 6) Measure OD of the supernatant at 595nm.

#### RFLP analysis

- 1) 1.0mL of liquid culture is transferred to a sterile micro tube.
- 2) Centrifuge at 13200 rpm (16000 g's) for 1 minute.
- 3) Discard the supernatant. Add another 1.0 mL of liquid culture into the same sterile micro tube.
- 4) Centrifuge at 13200 rpm (16000 g's) for 1 minute.
- 5) Discard the supernatant.
- 6) Follow QIAprep spin mini kit protocol for plasmid isolation.
- 7) Check DNA concentration using BioPhotometer.
- 8) Use Sac I and Kpn I for first double digestion. Incubate at 37 °C for 1 hour.
- 9) Use Pst I and Xho I for second double digestion. Incubate at 37 °C for 1 hour.
- 10) Add Loading dye into the digested sample.
- 11) Run the digested sample on a 1.0% agarose gel for 1-1.5 hours at 120 volts.
- 12) View gel with gel imager. All clones with a different digest pattern will be sent for sequence analysis.

### **EXAMPLE 7**

#### **ASSAY FOR AMYLASES**

#### **Preparation Of Host Cultures**

1. Start an overnight culture of XL1-Blue MRF' host cells. Use a single colony from a streak plate to inoculate 10 mL LB supplemented with 20 ug/mL tetracycline. Grow overnight culture shaking at 37°C for at least 16 hours.
2. Using aseptic technique, inoculate a fresh 100 mL of LB<sub>Tet</sub> day culture with XL1-Blue MRF' host from the overnight LB<sub>Tet</sub> culture.
3. Grow in a 37°C shaker until the OD reaches 0.75 – 1.0.
4. Pellet host cells at 1000 x g for 10 minutes and gently resuspend in 10 mM MgSO<sub>4</sub> at OD5.
5. Dilute a small amount of host cells to OD1 for use in titering and pintooling.
6. Host preparations can be used for up to 1 week when stored on ice or at 4°C.

#### COMMENTS

- To shorten growth time for the day culture, use ½X the usual Tet concentration in LB (½X = 10 ug/mL), or omit the antibiotic altogether.
- Do not use NZY when selecting with Tetracycline. The high Mg<sup>++</sup> concentration in NZY medium renders Tet inactive.

#### Titering Lambda Libraries

7. Place three sterile microfuge tubes in a rack.
8. Aliquot 995 uL prepared host cells in one tube and 45 uL prepared OD1 host cells into each of the two remaining tubes.
9. Add 5 uL of lambda library to the tube containing 995 uL host cells and mix by vortexing. This results in a dilution factor of 200.
10. Prepare 1/2,000 and 1/20,000 dilutions by consecutively adding 5 uL of previous dilution to the remaining two tubes containing 45 uL prepared host cells. Mix by vortexing after each dilution was made.
11. Allow phage to adsorb to host by incubating at 37°C for 15 minutes.
12. Meanwhile, pipet 100 uL of prepared OD1 host cells to each of three Falcon 2059 tubes.
13. Add 5 uL of each dilution to a separate 2059 tube containing host cells.

14. Plate each by adding 3 mL top agar to each tube and quickly pour over 90 mm NZY plates. Ensure a smooth, even distribution before the top agar hardens.
15. Invert plates and incubate at 37°C overnight.
16. Count plaques and calculate titer of the library stock (in plaque forming units (pfu) per uL).

### **Lambda Microtiter Screening For Amylases**

#### **Preparation**

1. Prepare a sufficient amount of XL1-Blue MRF' host culture, as described above, for the amount of screening planned. A culture of 100 mL is usually sufficient for screening 2-3 libraries.
2. Autoclave several bottles compatible with the QFill2 dispenser. These are the wide-mouth Corning bottles, 250 mL containing a sealing ring around the lip.
3. Make sure there are sufficient amounts of plates, top agar, BODIPY starch, red starch solution, etc. available for the screen.
4. Schedule the Day 2 robot run with a representative from Automation.

#### **Day 1**

1. Label the 1536-well plates (black) with library screen and plate number. Tough-Tags™ tube stickers, cut in half width-wise, are ideal for labeling 1536 well plates.
2. Calculate volumes of library, host cells and NZY medium necessary for the screen. This is easily done with an Excel spreadsheet.
3. Combine the calculated volumes of lambda library and OD5 host cells in a sterile 250 mL wide-mouth Corning bottle (containing a sealing ring).
4. Allow adsorption to occur at 37°C for 15 minutes.

5. Add the calculated volume of NZY medium and mix well. This is referred to as the cell-phage-medium suspension.
6. Perform a concomitant titer by combining 50 uL of the cell-phage-medium suspension with 250 uL of OD1 host cells in a Falcon 2059 tube, then plating with 9 mL of top agar onto a 150 mm NZY plate. Incubate concomitant titer plate at 37°C overnight.
7. Load the dispenser with the remainder of the suspension and array each labeled 1536-well plate at 4 uL per well. If the dispenser leaves air bubbles in some wells, they can be removed by centrifuging the plates at 200 x g for 1 minute.
8. Add 0.5 uL of positive control phage to well position AD46 of at least two of the assay plates. Use a strong amylase-positive lambda clone for this purpose. The lambda versions of SEQ ID NO.: 113 or SEQ ID NO.: 199 are good choices for positive controls.
9. Incubate assay plates at 37°C overnight in a humidified ( $\geq 95\%$ ) incubator.

Day 2

1. Count the pfu on the concomitant titer plate and determine the average seed density per well (in pfu per well).
2. Pintool at least 2 plates of each library screen (preferably the 2 containing positive controls) as follows:
  - a) Prepare 2 host lawn plates to act as a surface on which to pintool: combine 250 uL of OD1 host cells with 2 mL 2% red starch and plate with 9 mL top agar onto 150 mm NZY plates. Hold each plate as level as possible as the top agar solidifies in order to produce an even hue of red across the plate.
  - b) Using a twice flame-sterilized 1536 position pintool, replicate at least 2 of the screening plates onto the host lawn plates.
  - c) Place the pintoled recipient plates in a laminar flow hood with the lids off for about 15-30 minutes (to vent off excess moisture).
  - d) Replace the lids and incubate inverted at 37°C overnight.

3. Prepare the 2X BODIPY starch substrate buffer as follows:
  - a) Calculate the total volume of 2X substrate buffer solution needed for all screening plates at 4 uL per well (including any extra deadspace volume required by the dispenser) and measure this amount of 100 mM CAPS pH 10.4 into a vessel appropriate for the dispenser used.
  - b) Retrieve enough 0.5 mg tubes of BODIPY starch to produce the required volume of 2X substrate buffer [calculated in step a) above] at a final concentration of 20-30 ug/mL.
  - c) Dissolve each 0.5 mg tube in 50 uL DMSO at room temperature, protected from light, with frequent vortexing. This takes more than 15 minutes; some production lots of BODIPY starch dissolve better than others.
  - d) Add 50 uL 100mM CAPS buffer pH 10.4 to each tube and mix by vortexing.
  - e) Pool the contents of all tubes and remove any undissolved aggregates by centrifuging for 1 minute at maximum speed in a microfuge.
  - f) Add the supernatant to the rest of the 100 mM CAPS buffer measured in step a) above.
  - g) Protect the 2X substrate buffer from light by wrapping in foil.
4. Take plates and substrate buffer to the automation room and program the robot with the following parameters:
  - a) dispense 4 uL substrate buffer per well.
  - b) 1<sup>st</sup> read at 1 hour post-substrate, 2<sup>nd</sup> read at 9 hours, and third read at 17 hours; with 37°C incubation between reads
  - c) excitation filter: 485 nm; emission filter: 535 nm
  - d) set the Spectrafluor gain at 70, or the optimal gain for the batch of 2X substrate buffer prepared.
  - e) ensure that the incubator used will protect assay plates from light.

Day 3

1. Check pintoled plates for clearings in the bacterial lawn at all positions corresponding to wells on the associated assay plate. Also check for clearings in the red starch in any of the pin positions. If plates containing positive controls were used

for pintothing, you should be able to see a large clearing zone in the red background. Be wary of contaminants that also form clearing zones in red starch (see comment "Contaminants That Form Clearing Zones in Red Starch" at end of Example 7).

2. Identify putative hits from the data file produced by the robot computer. The KANAL program produced by Engineering simplifies data analysis. As a rule of thumb, a putative hit is characterized as a well having signal intensity rising at least 1.5 fold over background.
3. For each putative, remove 2 uL from the well and add to a tube containing 500 uL SM buffer and 50 uL CHCl<sub>3</sub>. Vortex to mix and store at 4°C. This solution will be referred to hereafter as the 4e-3 stock. The original screening plates should be stored at 4°C, protected from light, at least until breakouts are complete.

This is the recommended method of breaking out putative hits. It is a liquid phase assay that relies on confirmation of activity on BODIPY starch. Alternatively, putative hits can be plated directly onto solid phase plates containing red starch such that 2,000-3,000 pfu per hit are examined for clearing zones. However, inability to observe clearing zones on red starch is not necessarily an indication that a putative hit was a false positive. It would then need to be assayed using the format in which it was originally identified (i.e., liquid phase using BODIPY starch as substrate). In addition, very weak positives are more easily identified using the method detailed below.

Day 1

1. In a sterile 50 mL conical tube, combine 0.5 mL OD5 host cells with 45.5 mL NZY. This will be referred to as the host-medium suspension.
2. For each putative hit to be analyzed, aliquot 1 mL of host-medium suspension into each of 3 three sterile microfuge tubes.
3. Set the 12-channel pipetman in multidispense mode with an aliquot size of 20 uL and an aliquot number of 2x. Mount the pipetman with a clean set of sterile tips.
4. Pour about 1 mL of host-medium suspension into a new sterile solution basin and load the multichannel pipetman.
5. Dispense 20 uL per well into the last row (row P) of a black 384-well plate (12 channels x 2 = 24 wells). This row will be used later for the controls.

6. Expel the remaining liquid in the tips by touching the tips against the surface of the basin and pressing the RESET button on the pipetman. Lay the pipetman down in a way to prevent contamination of the tips. There is no need to change the tips at this point.
7. Pour the remainder of the fluid in the basin into a waste container (like a beaker) taking care to avoid splash-back contamination.
8. For the first putative to be analyzed, take 111 uL of the  $4e-3$  stock (see Day 2 in *Lambda Microtiter Screening for Amylases*) and add it to the first in a set of three tubes containing 1 mL host-medium suspension (step 2). Vortex to mix. This is *Dilution A*.
9. Take 111 uL of Dilution A and add to the next tube in the set. Vortex to mix. This is *Dilution B*.
10. Take 111 uL of Dilution B and add to the last tube in the set. Vortex to mix. This is *Dilution C*. You should now have three dilutions of phage, where concentrations of each differ by a factor of 10.
11. Pour the contents of Dilution C (the most dilute of the 3 samples) into the solution basin and load the multichannel pipetman.
12. Dispense 20 uL per well into the first row of the 384-well plate (12 channels  $\times$  2 = 24 wells).
13. Expel the remaining liquid in the tips by touching the tips against the surface of the basin and pressing the RESET button on the pipetman. Lay the pipetman down in a way to prevent contamination of the tips. There is no need to change the tips at this point.
14. Empty the basin as described above.
15. Pour the contents of Dilution B into the same basin and load the multichannel pipetman.
16. Dispense 20 uL per well into the second row of the 384-well plate.
17. Perform steps 13-16 similarly to dispense Dilution A into the third row of the plate.
18. After all three dilutions have been arrayed into the first 3 rows of the plate, discard all tips and the solution basin into the biohazardous waste container.
19. Mount the pipetman with a clean set of sterile tips and open a new sterile solution basin.

20. Repeat steps 8-19 for each remaining putative hit, using remaining rows on the plate up to row O. Five putative hits can be analyzed on one 384-well plate, with the last row (row P) saved for the controls.
21. Add 0.5 uL of each control to a separate well. Use at least 2-3 separate controls, preferably covering a range of activity.
22. Incubate assay plates at 37°C overnight in a humidified ( $\geq 95\%$ ) incubator.

#### Day 2

1. Pintool all breakout plates onto a host lawn with red starch using the same method described for Day 2 in *Lambda Microtiter Screening for Amylases*, except that a 384 position pintool is used.
2. Prepare the 2X BODIPY starch substrate buffer as follows:
  - a) Calculate the total volume of 2X substrate buffer solution needed for all breakout plates at 20 uL per well (including any extra deadspace volume required by the dispenser) and measure this amount of 100 mM CAPS pH 10.4 into a vessel appropriate for the dispenser used.
  - b) Retrieve enough 0.5 mg tubes of BODIPY starch to produce the required volume of 2X substrate buffer [calculated in step a) above] at a final concentration of 20-30 ug/mL.
  - c) Dissolve each 0.5 mg tube in 50 uL DMSO at room temperature, protected from light, with frequent vortexing. This takes more than 15 minutes; some production lots of BODIPY starch dissolve better than others.
  - d) Add 50 uL 100mM CAPS buffer pH 10.4 to each tube and mix by vortexing.
  - e) Pool the contents of all tubes and remove any undissolved aggregates by centrifuging for 1 minute at maximum speed in a microfuge.
  - f) Add the supernatant to the rest of the 100 mM CAPS buffer measured in step a) above.
  - g) Protect the 2X substrate buffer from light by wrapping in foil.
3. Dispense 20 uL per well into all breakout plates.
4. Wrap all plates in aluminum foil and incubate at room temperature for 2-6 hours.
5. Read each plate in the Spectrafluor with the following settings:



- a) fluorescence read (excitation filter: 485 nm; emission filter: 535 nm)
  - b) plate definition: 384 well black
  - c) read from the top
  - d) optimal gain
  - e) number of flashes: 3
6. On the resulting Excel spreadsheet, chart each putative's 3 rows in a separate graph and check for activity. Ensure that the positives controls produced signals over background.
  7. For each putative that appears to have a real signal among the wells, harvest a sample from a positive well as follows:
    - a) Select a positive well from a row representing the highest initial dilution.
    - b) Transfer 2 uL from that well into a tube containing 500 uL SM and 50 uL  $\text{CHCl}_3$ . This is referred to as the breakout stock.
    - c) Store at 4°C.
  8. Using methods previously described, plate about 10 uL of each breakout stock onto 150 mm NZY plates using red starch. The objective is to obtain several (at least 20) well-separated plaques from which to core isolates.

### Day 3

1. Check pintoed plates for an acceptable incidence of clearings in the bacterial lawn corresponding to wells on the associated assay plate. Also check for clearings in the red starch in the positive controls and in any tested putatives. Be wary of contaminants that also form clearing zones in red starch (see below).
2. From the solid phase plates containing dilutions of breakout stocks, core several isolated plaques, each into 500 uL SM with 50 uL  $\text{CHCl}_3$ . This is referred to as the isolate stock.
3. The isolate stocks can then be individually tested on BODIPY starch using methods described above. This step can be skipped if the plaque that was cored in step 2 produced a clearing zone in the red starch background. The isolate stocks were then be individually tested on BODIPY starch using methods described above. However,

this step may be skipped if the plaque that was cored in step 2 produced a clearing zone in the red starch background.

### **Excisions**

#### **Day 1**

1. In a Falcon 2059 tube, mix 200 uL OD1 XL1-Blue MRF' host, 100 uL lambda isolate stock and 1 uL ExAssist phage stock.
2. Incubate in 37°C shaker for 15 minutes.
3. Add 3 mL NZY medium.
4. Incubate in 30°C shaker overnight.

#### **Day 2**

1. Heat to excision tube to 70°C for 20 minutes.
2. Centrifuge 1000 x g for 10 minutes.
3. In a Falcon 2059 tube, combine 50 uL supernatant with 200 uL EXP505 OD1 host.
4. Incubate in 37°C shaker for 15 minutes.
5. Add 300 uL SOB medium.
6. Incubate in 37C shaker for 30-45 minutes.
7. Plate 50 uL on large LB<sub>Kan50</sub> plate using sterile glass beads. If the plates are "dry", extra SOB medium can be added to help disburse the cells.
8. Incubate plate at 30°C for at least 24 hours.
9. Culture an isolate for sequencing and/or RFLP.

Growth at 30°C reduces plasmid copy number and is used to mitigate the apparent toxicity of some amylase clones.

### **Contaminants That Form Clearing Zones in Red Starch**

When using red starch on solid medium to assay phage for amylase activity, it is common to see contaminating colony forming units (cfu) that form clearing zones in the red

starch. For pintoed plates, it is important to distinguish amylase-positive phage clones from these contaminants whenever they align with a particular well position. The source of the contaminating microbes is presumably the 2% red starch stock solution, which cannot be sterilized by autoclaving or by filtering after preparation. It is thought that they are opportunistic organisms that survive by metabolizing the red starch. In order to reduce these contaminants, use sterile technique when making 2% red starch solutions and store the stocks either at 4°C or on ice.

### EXAMPLE 8

#### BIOINFORMATIC ANALYSIS

An Initial bioinformatic analysis was made with the known hyperthermophilic  $\alpha$ -amylase sequences. Figure 14a shows an alignment of the sequences some of which have been deposited at the NCBI database. This analysis revealed the potential for designing degenerate primers to PCR the entire gene minus its signal sequence (see Figure 14a), yielding potentially novel full-length alpha amylases from a library.

The following libraries were screened by PCR from genomic DNA:

Table 6:

Library #	Name	PCR positive	Subcloned
5	<i>A.lithotropicus</i>	No	
13	<i>Pyrodictium occultum</i>	No	
17	<i>Pyrodictium TAG11</i>	No	Yes
113	<i>Deep sea enrichment</i>	Yes	Yes
170	<i>Deep sea enrichment</i>	Yes	Yes
198	<i>Archaeoglobus</i>	No	
206	<i>Acidianus sp</i>	No	
453	<i>Mixed iceland enrich</i>	No	
455	<i>Mixed iceland enrich</i>	Yes	Yes

Figure 14b shows an alignment of the identified sequences and the table below lists their relative percent identities.

**Table 7: Nucleotide sequence % identity**

	SEQ ID NO.: 81	pyro	Pyro 2	thermo	therm2	SEQ ID NO.: 75	SEQ ID NO.: 77	SEQ ID NO.: 83	SEQ ID NO.: 85	SEQ ID NO.: 79	Clone A
SEQ ID NO.: 81	100	91.7	75.1	82.1	80.1	82.5	82.6	82.1	82.6	83	77.8
pyro		100	74.8	82.5	80.5	82	82.2	82.9	82.8	84	78.5
Pyro2			100	71.5	71.1	74	74.2	77	77.1	73	70.5
therm				100	81.7	83.5	83.8	82.8	83.2	83.8	76.4
therm2					100	88.9	88.8	84.1	84.7	84	76.3
SEQ ID NO.: 75						100	98.3	84.6	85.2	85.5	77
SEQ ID NO.: 77							100	84.8	84.9	85.4	77.4
SEQ ID NO.: 83								100	96	83.3	78.5
SEQ ID NO.: 85									100	83	78.1
SEQ ID NO.: 79										100	79.8
Clone A											100

The amino acid identity ranges from about 85-98% identity. Accordingly, these sequences are useful in shuffling of genes as described herein.

Figure 14c shows the nucleic acid alignment of the corresponding polypeptide sequences above. Expression of these amylases in the expression vector pSE420 and the host cell line XL1-Blue showed 1703 and 1706 to have amylase activity.

#### **EXAMPLE 9**

#### **Characterization of Library 63 GP-1 alpha amylase pH optimum and specific activity determination**

In initial experiments, the SEQ ID NO: 81 from *Thermococcus* showed that it was effective in both starch liquefaction for corn wet milling and desizing for textiles. This enzyme has a pH optimum of 4.5 to 5.0. At this lower pH, it is possible to use little or no calcium which lowers overall operating costs and less byproduct formation. In addition, at this low pH, there is decreased chemical usage and ion exchange load. The industry standard *B. licheniformis* amylase is suboptimal in both thermostability and pH optimum. The 63GP-1 amylase has a higher application specific activity compared to *B. licheniformis* amylase and therefore much less enzyme is required to hydrolyze a ton of starch (as much as 20-fold less enzyme can be used).

The pH optimum for the hydrolysis of starch was determined by reacting 50 uL of the GP-1, 0.35 U/ml, with a 100ml of 1% soluble starch solution (0.0175U/g of starch) for 30 minutes at 95 degrees C. The reducing ends generated in the liquefied starch solution were measured by the neocupronine assay, described herein. The percent hydrolysis of cornstarch was determined by measuring the number of sugar reducing ends produced with the neocupronine assay. Seventy grams of buffer solution (pH4-7) was weighed and 100ppm of calcium was added. Thirty grams of cornstarch was mixed into the buffer solution to form a starch slurry. The enzyme was added and the vessels sealed and incubated at 95 degrees C for 30 minutes with an initial heating rate of six degrees C per minute. A 1 ml sample was extracted from the reaction beakers and analyzed by the neocupronine assay. The optimum for GP-1 was between pH 4.5 and 5 , while the commercial *B. licheniformis* amylase performed optimally at about pH 6.0.

#### **EXAMPLE 10**

##### **Amylase Ligation Reassembly**

Nine fragments (each about 150 bp) were amplified from each of the parent clones SEQ ID NO.: 81, SEQ ID NO.: 77, SEQ ID NO.: 79, covering the whole open reading frame. The primers are provided in Table 8.

TABLE 8

	SEQ ID NO:	
GAACACTAGTAGGAGGTAACCTTATGGCAAAGTATTCCGAGCTCGAAG	258	SpeI
GAACGGTCTCATTCCGCCAGCCAGCAAGGGGATGAGCGG	259	BsaI
GAACCGTCTCAAAACACGGCCCATGCCTACGGC	260	BsmBI
GAACGTCTCACCTCGACTTCCACCCCAACGAGGTCAAG	261	BsmAI
GAACGTCTCAGGCGCTTTGACTACGTGAAGGGC	262	BsmAI
GAACGGTCTCAACAAGATGGATGAGGCCTTTG	263	BsaI
GAACCGTCTCACGATATAATCTGGAACAAGTACCTTGC	264	BsmBI
GAACCGTCTCAGAAGCACGAGCATAGTTTACTACG	265	BsmBI
GAACCGTCTCAAAGGTGGGTTTATGTGCCG	266	BsmBI
GAACGTCTCAGGAATCCAAATGGCGGATATTCCCGC	267	BsmAI
GAACGGTCTCAGTTTATCATATTGATGAGCTCC	268	BsaI
GAACCGTCTCAGAGGTAGTTGGCAGTATATTTG	269	BsmBI
GAACGTCTCACGCCAGGCATCAACGCCGATG	270	BsmAI
GAACGTCTCA TTGTAGTAGAGCGGGAAGTC	271	BsmAI
GAACGGTCTCAATCGGTGTCTGTGGTTTGCTAC	272	BsaI
GAACCGTCTCACTTCCACCTGCGAGGTGGTC	273	BsmBI
GAACCGTCTCACCTTCCAACTTGCTCGAGC	274	BsmBI
TCGAGACTGACTCTCACCCAACACCGCAATAGC	275	
GAACACTAGTAGGAGGTAACCTTATGGCCAAGTACCTGGAGCTCGAAG	276	SpeI
AGG		
GAACGGTCTCATTCCCCCGGCGAGCAAGGGC	277	BsaI
GAACCGTCTCAAAACACCGCCCACGCCTACGG	278	BsmBI
GAACGTCTCACCTCGACTTCCACCCCAAC	279	BsmAI
GAACGTCTCAGGCGCTTCGACTACGTCAAGG	280	BsmAI
GAACGGTCTCAACAAGATGGACGCGGCCTTTGAC	281	BsaI
GAACCGTCTCACGATATAATTTGGAACAAGTACCC	282	BsmBI
GAACCGTCTCAGAAGCACCGACATAGTCTAC	283	BsmBI
GAACCGTCTCAAAGGTGGGTCTACGTTCCG	284	BsmBI
GAACGTCTCAGGAATCCATATTGCGGAGATTCCGGC	285	BsmAI
GAACGGTCTCAGTTTATCATGTTACGAGCTC	286	BsaI
GAACCGTCTCAGAGGTAGTTGGCCGTGTACTTG	287	BsmBI
GAACGTCTCAGCCATGCGTCAACGCCGATG	288	BsmAI
GAACGTCTCA TTGTAGTAGAGCGGGAAGTCG	289	BsmAI
GAACGGTCTCAATCGGTGTCTGTGGTTTGCAACG	290	BsaI
GAACCGTCTCACTTCCACCGGCGAGGTGGTCGTG	291	BsmBI
GAACCGTCTCACCTTCCGGCCTTGCTCGAGCC	292	BsmBI
TCGAGACTGACTCTCAGCCCACCCCGCAGTAGCTC	293	
GAACACTAGTAGGAGGTAACCTTATGGCCAAGTACTCCGAGCTGGAAG	294	SpeI
AGG		
GAACGGTCTCATTCCCTCCCGCGAGCAAGGG	295	BsaI
GAACCGTCTCAAAACACCGCCCACGCCTATG	296	BsmBI
GAACGTCTCACCTCGACTTCCACCCGAACGAGC	297	BsmAI
GAACGTCTCAGGCGCTTCGACTACGTCAAGG	298	BsmAI
GAACGGTCTCAACAAGATGGACGAGGCCTTCG	299	BsaI
GAACCGTCTCACGATATAATCTGGAACAAG	300	BsmBI
GAACCGTCTCAGAAGCACTGACATCGTTTACTACG	301	BsmBI
GAACCGTCTCAAAGGTGGGTTTACGTTCCG	302	BsmBI
GAACGTCTCAGGAATCCATATCGCCGAAAT	303	BsmAI

GAACGGTCTCAGTTTATCATGTTTATGAGC	304	BsaI
GAACCGTCTCAGAGGTAGTTGGCCGTGTATTAC	305	BsmBI
GAACGTCTCAGCCAGGCATCGATGCCGAT	306	BsmAI
GAACGTCTCATTGTAGTAGAGGGCGAAGTCAAAG	307	BsmAI
GAACGGTCTCAATCGGTATCGTGGTTGGCTACAAAC	308	BsaI
GAACCGTCTCACTTCCTCCGGCGAGGTTGTCATG	309	BsmBI
GAACCGTCTCACCTTCCGGCTTTGCTTGAGGC	310	BsmBI
TCGAGACTGACTCTCACCCAACACCGCAGTAGCTCC	311	
CACACAGCAGCAACCAACCTCGAGACTGACTCTCASC	312	BbvI

Conditions used for PCR were as follows: 3 min 94°C, (30 sec 94°C; 30 sec 55°C, 30 sec 68°C) x 30 cycles, followed by 10 min 68°C. PCR products corresponding to homologous regions from the three parents were pooled (1:1:1), cut with the appropriate restriction enzyme (see Table 8), and gel-purified. Equal amounts of fragment pools were combined and ligated (16 °C; over night). The resulting 450 bp ligation products were gel purified and ligated to yield full length amylase genes. The resulting full length products were gel-purified and PCR amplified using a mixture of F1 primers SEQ ID NO.: 81, SEQ ID NO.: 77, SEQ ID NO.: 79 and primer (SEQ ID NO: 312). Conditions used for PCR were as follows: 3 min 94°C, (30 sec 94°C; 30 sec 50°C, 60 sec 68°C) x 30 cycles, followed by 10 min 68°C. The resulting PCR products (~1.4 kbp) were purified, cut with *SpeI* and *BbvI*, gel-purified, ligated into pMYC (vector from Mycogen, cut with *SpeI/XhoI*), and transformed into *E.coli* Top10. Plasmid DNA from a pool of ~21000 colonies was isolated and transformed into *Pseudomonas*.

#### **Screening of Reassembled $\alpha$ -amylase**

The transformed *Pseudomonas fluorescens* (MB214) containing pMYC derived from the parent clones SEQ ID NO.: 81, SEQ ID NO.: 77, SEQ ID NO.: 79 were sorted to 96- or 384-well plates by FACS and treated with 6M urea. Primary screening using RBB-starch and/or FITC-starch as substrates was carried out as described more fully below. Elevated active clones were screened using RBB-starch as substrate using induced cultures and by liquefaction assay. Stock and sequencing new elevated active clones based on liquefaction data was performed.

The transformed reassembled amylase library (MB214 (Pf)), were collected and sorted into 96-well plates (or 384-well plates) at 1 cell/well in 50µl of LB+Tet. The plates

were incubated for 24 hours at 30°C. Replicate plates were made corresponding to each well for storage. Forty-five (45) µl of 12M urea was added to each well and the plates were shaken for 10 minutes. Plates were kept at room temp for at least 1 hour and the lysate stored at 4°C.

#### **Assay Using RBB-starch**

75µl of RBB-starch substrate (1% RBB-insoluble corn starch in 50mM NaAc buffer, pH=4.5) was added into each well of a new 96-well plate (V-bottom). Five micro-liters of enzyme lysate was transferred into each well with substrate using Biomek or Zymark. The plates were sealed with aluminum sealing tape and shaken briefly on the shaker. The plates were incubated at 90°C for 30 minutes, followed by cooling at room temperature for about 5 to 10 minutes. One hundred micro-liters of 100% ethanol was added to each well, the plates sealed and shaken briefly on the shaker. The plates were then centrifuged 4000rpm for 20 minutes using bench-top centrifuge. 100µl of the supernatant was transferred into a new 96-well plate (flat bottom) by Biomek and read OD<sub>595</sub>. Controls: SEQ ID NO.: 81, SEQ ID NO.: 77, SEQ ID NO.: 79.

#### **Assay using FITC-starch**

Added 50µl of substrate (0.01% FITC-starch in 100mM NaAc buffer, pH=4.5) into each well of a new 384-well plate. Transferred 5µl of enzyme lysate into each well with substrate and incubated the plate at room temperature overnight. The polarization change of the substrate, excitation 485nm, emission 535nm, was read for each well. Controls: SEQ ID NO.: 81, SEQ ID NO.: 77, SEQ ID NO.: 79. Preferably 96 well plates are used for all assays.

#### **Confirmation of new active clones**

Each positive clone from screening was grown and induced using a standard protocol. Each clone was examined for growth (i.e., cell density over time), activity at per cell level (RBB-starch assay and liquefaction assay), expression (protein gel) and solubility of protein (by microscope analysis). The confirmed new elevated clones were transferred for fermentation.



Table 3

<u>SEQ ID NO.</u>	<u>Signal Sequence</u>
SEQ ID NO: 87	AA1-23 (SEQ ID NO:213)
SEQ ID NO: 91	AA1-23 (SEQ ID NO: 214)
SEQ ID NO: 93	AA1-33 (SEQ ID NO: 215)
SEQ ID NO: 97	AA1-31 (SEQ ID NO: 216)
SEQ ID NO: 99	AA1-30 (SEQ ID NO: 217)
SEQ ID NO: 103	AA1-22 (SEQ ID NO: 218)
SEQ ID NO: 105	AA1-33 (SEQ ID NO: 219)
SEQ ID NO: 109	AA1-25 (SEQ ID NO: 220)
SEQ ID NO: 111	AA1-35 (SEQ ID NO: 221)
SEQ ID NO: 113	AA1-28 (SEQ ID NO: 222)
SEQ ID NO: 117	AA1-21 (SEQ ID NO: 223)
SEQ ID NO: 119	AA1-30 (SEQ ID NO: 224)
SEQ ID NO: 123	AA1-35 (SEQ ID NO: 225)
SEQ ID NO: 125	AA1-28 (SEQ ID NO: 226)
SEQ ID NO: 127	AA1-30 (SEQ ID NO: 227)
SEQ ID NO: 131	AA1-30 (SEQ ID NO: 228)
SEQ ID NO: 133	AA1-30 (SEQ ID NO: 229)
SEQ ID NO: 137	AA1-28 (SEQ ID NO: 230)
SEQ ID NO: 139	AA1-23 (SEQ ID NO: 231)
SEQ ID NO: 141	AA1-23 (SEQ ID NO: 232)
SEQ ID NO: 143	AA1-30 (SEQ ID NO: 233)
SEQ ID NO: 145	AA1-27 (SEQ ID NO: 234)
SEQ ID NO: 147	AA1-29 (SEQ ID NO: 235)
SEQ ID NO: 149	AA1-28 (SEQ ID NO: 236)
SEQ ID NO: 69	AA1-27 (SEQ ID NO: 237)
SEQ ID NO: 153	AA1-26 (SEQ ID NO: 238)
SEQ ID NO: 155	AA1-33 (SEQ ID NO: 239)
SEQ ID NO: 157	AA1-25 (SEQ ID NO: 240)
SEQ ID NO: 159	AA1-25 (SEQ ID NO: 241)
SEQ ID NO: 161	AA1-36 (SEQ ID NO: 242)
SEQ ID NO: 167	AA1-36 (SEQ ID NO: 243)
SEQ ID NO: 169	AA1-23 (SEQ ID NO: 244)
SEQ ID NO: 173	AA1-25 (SEQ ID NO: 245)
SEQ ID NO: 175	AA1-22 (SEQ ID NO: 246)
SEQ ID NO: 177	AA1-23 (SEQ ID NO: 247)
SEQ ID NO: 179	AA1-23 (SEQ ID NO: 248)
SEQ ID NO: 185	AA1-25 (SEQ ID NO: 249)
SEQ ID NO: 189	AA1-36 (SEQ ID NO: 250)
SEQ ID NO: 191	AA1-25 (SEQ ID NO: 251)
SEQ ID NO: 193	AA1-25 (SEQ ID NO: 252)
SEQ ID NO: 197	AA1-23 (SEQ ID NO: 253)
SEQ ID NO: 199	AA1-23 (SEQ ID NO: 254)
SEQ ID NO: 201	AA1-30 (SEQ ID NO: 255)
SEQ ID NO: 203	AA1-25 (SEQ ID NO: 256)
SEQ ID NO: 205	AA1-16 (SEQ ID NO: 257)
SEQ ID NO.:73	AA1-16 (SEQ ID NO: 7)
SEQ ID NO.: 79	AA1-26 (SEQ ID NO: 8)